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ANNALS OF BOTANY

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With twelve Plates and
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A Quantitative Study of the Early Development of the Seedling of Cacao (*Theobroma cacao*)

BY

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With ten Figures in the Text

INTRODUCTION

IN the course of investigations of the growth rate of the cacao seedling it became of interest to know to what extent the plant depended upon the cotyledons for the supply of material, at what stage it became independent of the reserves stored in the seed, how much of the seed reserves were lost in respiration before this occurred, and in what manner the material translocated from the cotyledons and formed by assimilation was distributed among the various other organs of the seedling as development proceeded.

Quantitative data on this phase of plant development are remarkably scanty in the literature. During the latter part of last century, and to a less extent more recently, a considerable number of investigators studied the course of accumulation of dry matter in annual crop plants, and sometimes also its distribution among different organs. In general, however, the first harvest was taken well after the plant had become self-supporting and showed a very considerable increase in dry weight over that of the seed.

Cacao lends itself to studies of early growth more than most species of crop plants. The large seed, prompt epigeal germination, and slow early development facilitate detailed determinations of dry-weight changes. It should be noted, however, that cacao differs markedly from most temperate plants in that the leaves develop in 'flushes'. Instead of the expansion of successive leaves of the shoot following in regular sequence, a few leaves, commonly about four, expand almost simultaneously, and an interval of several weeks or months then intervenes before expansion of new leaves occurs again. The observations to be described here, however, extend only to the time of 'hardening' (maturation) of the first 'flush' of leaves.

While growth observations of the type to be described can never have direct relevance to a broader field than the actual species investigated, the fact that such detailed quantitative work on early seedling development appears not to have been attempted in other species will, it is hoped, give the investigation a measure of general interest.

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EXPERIMENTAL PROCEDURE

Experiment I

On November 5, 1946, eight seeds were planted in each of 250 baskets 10 in. in diameter. These baskets had been filled with topsoil taken from a cacao plantation, and were standing in a nursery under shade cast by trees of *Leucaena glauca*, where the light intensity was about 18 per cent. of full daylight. The beans for planting were taken from four Amelonado clones (TF₄, TF₅, TF₇, and TF₁₇), two beans from each clone being planted in each basket; the eight beans destined for each basket were weighed together before planting after the testas had been removed. The baskets were divided into 10 groups of 25 according to the time at which the beans planted in them had been prepared.

Four samples of eight beans were taken for initial dry-weight determinations at the same time as the rest were planted. The radicles were separated from the cotyledons, and radicles and cotyledons were weighed; they were dried for 24 hours at 100° C., and then again weighed.

By November 14, approximately half the seedlings had emerged, and the first harvest was taken. One basket was selected at random from each of the 10 groups of 25. The seedlings in each of these 10 baskets were separated from the soil, and the cotyledons were severed from the radicle and hypocotyl. The radicles were carefully washed in water and dried with blotting-paper, and the fresh weights of the organs were then obtained, the eight radicles and hypocotyls from a basket forming one sample, the 16 cotyledons another. The samples were then dried in an oven at 100° C. for at least 24 hours and reweighed. This harvesting procedure was repeated daily, with the exception that from November 16 the plumule was separated from the other organs (it had previously been too small to weigh separately), and from November 22 was divided into leaves and stem; from November 22, also, the leaf area in each basket was recorded, the smallest leaves being outlined on millimetre graph paper, the larger ones being printed on paper impregnated with methyl violet (Goodall, 1947) and subsequently measured by counting the half-centimetre squares on a glass grid covering the print.

Up to November 20 all baskets had been included in the randomization to determine which should be harvested on each occasion. After that date baskets in which one or more seedlings were severely retarded were excluded from harvest. The experiment was brought to an end on December 3 by lack of material.

Experiment II

The results of expt. I showed that its duration had hardly been adequate for the purpose intended, for at the time of the final harvest the plants had still not regained their initial dry weight. Moreover, it appeared that a substantial amount of dry matter had been lost by the seedling before emergence, so that it seemed desirable to commence the regular harvests from the date of planting rather than from the mean date of emergence. In order

to avoid increasing to any great extent the amount of material to be handled, it was accordingly decided in subsequent work to harvest at 2-day intervals.

A further difference between the first and subsequent experiments was occasioned by the smaller supply of planting material after the main crop had been harvested, so that it was not possible thereafter to plant the whole experiment with the progeny of four parents.

Experiment II was planted on December 30, 1946; 340 baskets were used, 34 complete baskets being planted with seed from each of 10 Amelonado parents. Again eight beans, previously weighed as a group, were planted in each basket, and the baskets were placed in the same nursery. Eight beans from each parent were taken on the day of planting as the first harvest, and thereafter on every second day one basket of seedlings from each parent was harvested. Up to and including the harvest of January 14 (by which date half the seedlings had emerged), each seedling was divided into radicle and cotyledons. On January 16 and 18 the plumule was also separated, and subsequently the seedlings were divided into four portions—cotyledons, root (including hypocotyl), stem, and leaves. In these harvests leaf areas were also determined. Fresh weights and dry weights were determined as in expt. I; unfortunately dry-weight data were not obtained for the harvest of January 22, since the material was destroyed by a failure in the oven.

Up to January 26 all baskets were included in the randomization prior to each harvest, but insect damage and failures in germination made it necessary to reject a number of baskets as from that date. The experiment concluded on February 15.

Experiment III

This experiment commenced in May, when planting material was even more difficult to obtain, and not more than two pods were obtainable from any one source. Accordingly, each basket received two beans from each of four Amelonado parent trees, the same four parent trees supplying planting material for 34 baskets. Ten such groups of baskets were planted on May 21, 1947, and 10 groups of 8 beans comparable to those planted were taken as the first harvest. As in the previous experiments each group of eight beans was weighed, without testas, before planting.

Harvests took place at intervals of 2 days, one basket of seedlings being taken from each of the 10 groups as in the previous experiment. Half the seedlings had emerged by May 31; the plumules could first be separated on June 2, and the leaves and stems 4 days later. All baskets were included in the randomization to determine which should be harvested up to June 12; insect damage, which was more severe in this experiment than formerly, and failure to germinate, made it necessary to restrict the randomization thereafter. The experiment terminated on July 2.

RESULTS

Dry-matter accumulation and distribution

The dry weights of the material harvested were tabulated by group and date of harvest, and the considerable influence of seed fresh weight was eliminated by an analysis of co-variance. The correlation (r) and regression (b) coefficients obtained in the interaction term are shown below:

	r	b	n
Experiment I . . .	0.829	0.657	170
Experiment II . . .	0.809	0.613	197
Experiment III . . .	0.839	0.620	188

The polynomial regression of the plant dry weight, corrected for seed fresh weight, on time was then computed. This was carried to the fifth term, though the fifth term itself did not prove significant in any of the three experiments. In the first and third experiments the regression thus computed (taken to the last significant term) failed to account for the whole of the excess of the variance between occasion means over the error term; nevertheless, in the second and third experiments a very large proportion of the total variance between occasion means was embodied in the regression. In the first experiment less than half of the variance between occasions was accounted for by the regression, this experiment covering a much shorter time than the others.

The coefficients of the successive terms in the regression are given in Table I, together with the variance ascribable to the regression (s_r^2), the residual variance between occasions (s_o^2) with its degrees of freedom (n_o), and the interaction variance from the original analysis (s_e^2)—which provides the best available estimate of error—also with the appropriate number of degrees of freedom (n_e). The time variable x is expressed in days from the mean date of emergence, and the plant dry weight in milligrams. The unit of data used in the analysis is the total weight of eight seedlings, but the figures in Table I, like all others quoted in this paper, are expressed in terms of a single seedling.

The data are also represented graphically in Figs. 1–3, the uppermost curve in each case representing the computed regression of the corrected values, the crosses the actual mean dry weight of the seedlings harvested on each occasion, irrespective of seed fresh weight.

The dry weight of each of the organs into which the plant had been divided was expressed as a percentage of that of the whole plant, and the variance due to group and time of harvest was separated from their interaction, which was treated as an estimate of error; the angular transformation was used for this analysis. The regression of the occasion means (still in angular transformation) on time was then computed, and the results are presented in Table I in the same way as for plant dry weight. In almost every case the regression accounts for well over 99 per cent. of the total variance between

TABLE I
Regression on Time of Plant Dry Weight and its Distribution among the Organs

Dependent variable.	Experiment	Coefficient of						s_e^2	n_e	s_e^2	n_e
		x^0	x	x^2 ($\times 10^3$)	x^3 ($\times 10^3$)	x^4 ($\times 10^6$)	x^5 ($\times 10^6$)				
Plant dry weight, corrected for seed fresh weight (mg.)	I	689.4	-31.10	+618.04	-440.14	+1,033.94	—	44,368	15	4,932	170
	II	870.8	-6.00	+24.40	+12.27	-29.13	—	1,375,919	18	8,348	107
	III	794.3	-7.78	+20.19	+21.35	-56.88	—	677,959	17	8,366	188
Cotyledons	I	74.8	-2.26	+23.66	-21.76	+59.35	—	33,032.95	15	12.74	171
	II	73.0	-1.34	+0.55	+0.98	-7.92	+1.75	121,602.85	17	15.76	108
	III	72.5	-1.66	+0.38	+2.49	-17.45	+3.31	108,707.41	16	26.31	189
Roots	I	15.6	+1.64	-5.39	—	—	—	12,158.97	17	4.80	171
	II	17.6	+1.30	-3.20	-2.59	+14.43	-1.85	28,409.08	17	5.10	108
	III	18.1	+1.78	-3.55	-5.45	+30.28	-4.28	31,573.34	16	15.32	189
Plumule	I	2.6	-0.30	+13.21	—	—	—	589.48	3	1.50	45
	II	0.0	+1.20	—	—	—	—	228.48	0	5.91	9
	III	1.5	+0.60	—	—	—	—	61.50	0	0.84	9
Stem	I	-2.8	+1.37	-2.71	—	—	—	2,345.32	9	3.58	99
	II	-0.1	+0.91	-1.14	—	—	—	8,381.80	10	4.14	108
	III	+12.0	+0.48	-12.09	—	—	—	8,900.61	11	8.10	117
Leaves	I	14.4	-4.62	+56.03	-14.63	—	—	18,422.60	8	26.95	99
	II	13.8	-4.25	+56.59	-20.35	+23.50	—	36,156.79	8	46.43	108
	III	19.2	-6.01	+71.20	-25.34	+29.49	—	36,147.39	9	11.07	117

sin⁻¹ (dry weight of organ/dry weight of plant)

occasions, but in a number of instances the residual variance between occasions remains significant.

These data are also represented graphically in Figs. 1-3. The percentages given by the regression equations have been multiplied by the computed values for plant dry weight to give the weights of the separate organs in milligrams. If adjustment of these organ dry weights was necessary in order

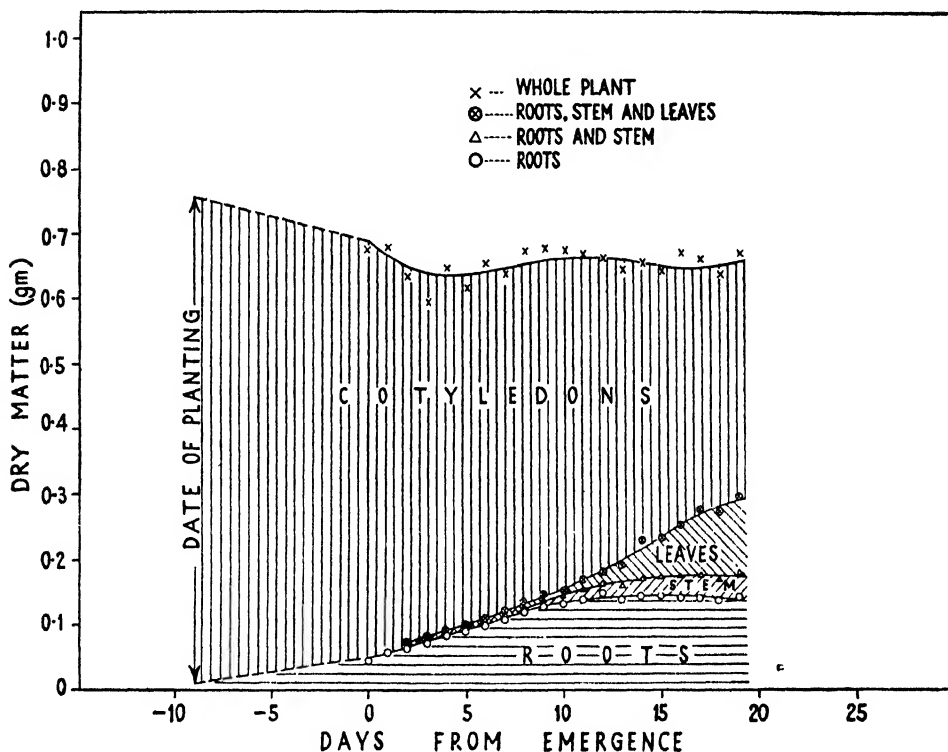


FIG. 1. Changes in distribution of dry matter in a single plant. Experiment I.

to give the correct total plant dry weight, the adjustment was distributed proportionately among them. The vertical distances between the curves in Figs. 1-3 represent the organ dry weights thus computed, while the vertical distances between the points shown represent the actual mean dry weights of the organs harvested on each occasion.

The general impression given by these curves indicates that the loss of dry matter by the seedling continues from the time of planting at least until the time when the cotyledons separate, some 3 or 4 days after the seedling has emerged from the soil. During this period about 15 per cent. of the original dry matter is lost—that is, the average rate of loss is about 1 per cent. per day; the rate increases, however, up to the time of emergence, when it has reached about 2 per cent. per day. The minimum plant dry weight occurs about 9 days after emergence, and the plant then begins to make up its losses

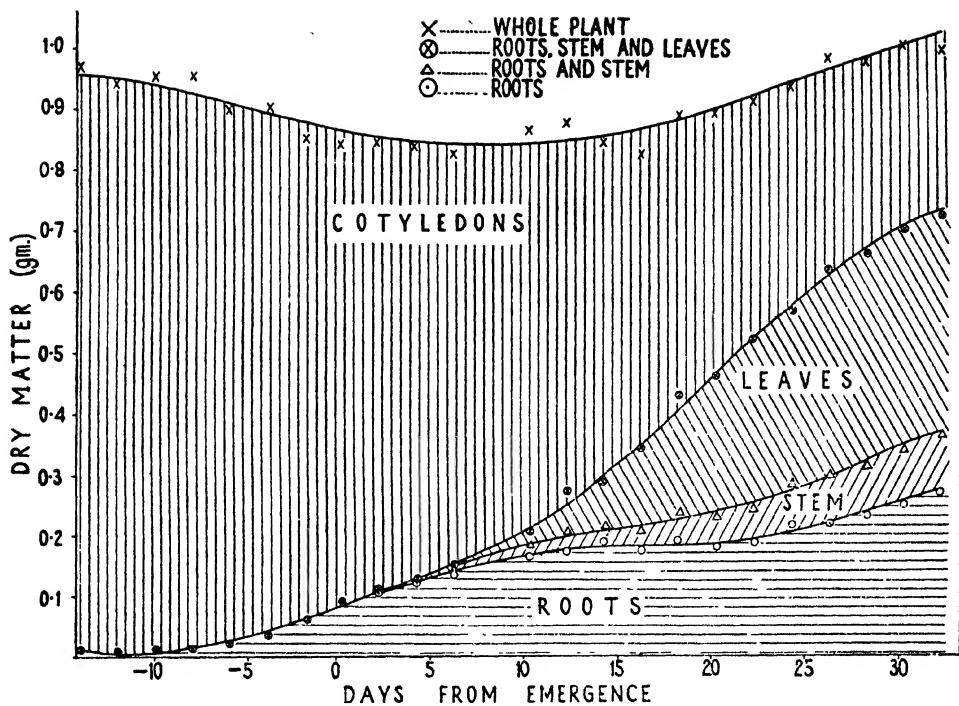


FIG. 2. Changes in distribution of dry matter in a single plant. Experiment II.

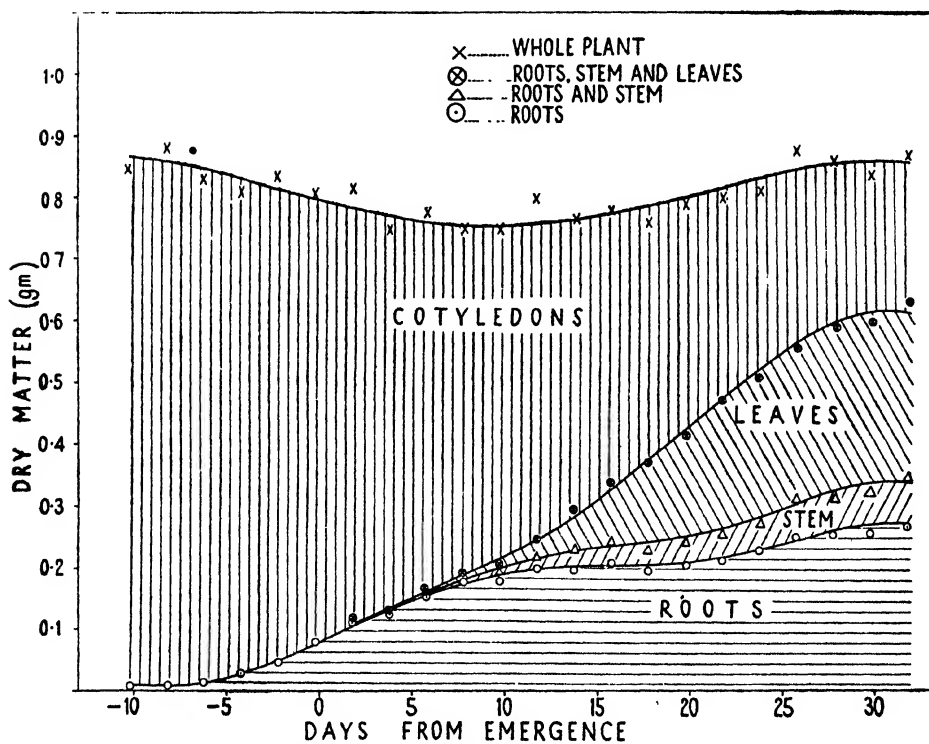


FIG. 3. Changes in distribution of dry matter in a single plant. Experiment III.

during germination, passing the initial seed dry weight about 4 weeks after emergence. It should be borne in mind that seedlings in early development often absorb mineral nutrients very actively (Godlewski, 1879), so that the losses through respiration may be considerably more than the net loss of 15 per cent. in dry matter. The relative growth rates (per cent. per day) during the third and fourth weeks after emergence amount to:

	Expt. II.	Expt. III.
Third week . . .	0.86	0.82
Fourth week . . .	1.14	0.81

These values are extremely low compared with those for other crop plants, the relative growth rates of which are commonly 10 per cent. per day or more (Goodall, 1945).

The approximate maximum net assimilation rates obtained from the regression curves, allowing 5 sq. cm. for the cotyledon area, are:

	Days from emergence.	Net assimilation rate (g./sq. dm./week).
Experiment I . . .	18	0.057
Experiment II . . .	19	0.062
Experiment III . . .	15	0.090

All these values are very low compared with those commonly recorded in the literature for other crops (Heath and Gregory, 1938)—often 0.5 g./sq. dm./week or more—but it must be remembered that the plants were grown under shade.

At the time of planting, the cotyledons contain nearly 99 per cent. of the embryo dry matter. Three or four days after planting, translocation of reserves to the radicle commences, and soon reaches a rate of 10 mg. (1.3 per cent. of the cotyledon dry weight) per day, excluding the amount used by the radicle in respiration. The rate of increase in the dry weight of the root becomes less when plumular development commences, and during the third week after emergence its dry weight appears to be almost stationary; its growth recommences, however, during the fourth week.

After the first week following emergence, the increase in dry matter of the stem proceeds very regularly at a rate of between 3 and 4 mg. per day. The rate of increase in the leaf dry matter is, on the other hand, very far from constant, reaching a maximum of some 20 mg. per day in the middle of the third week, and declining thereafter until by the end of the fourth week its increase has almost ceased. This is the time at which the first flush is hardening, and the terminal bud remains quiescent for some weeks before the second flush begins to expand.

Until the cotyledons separate, 3 or 4 days after emergence, the seedling is entirely dependent for its growth on the reserves contained in them. The upper surfaces of the cotyledons, however, form a little chlorophyll, and their assimilation doubtless contributes thereafter to the seedling's requirements, perhaps accounting in part for the fact that loss in dry weight by the plant as a whole ceases a few days later. Net loss in dry weight by the cotyledons,

however, continues fairly regularly, at least up to the end of the fourth week, by which time their weight is some 30 per cent. of that at the time of planting. From other observations, it may be added that the cotyledons are normally shed some 6 weeks after the seedling has emerged, still containing between 25 and 35 per cent. of the initial weight of dry matter.

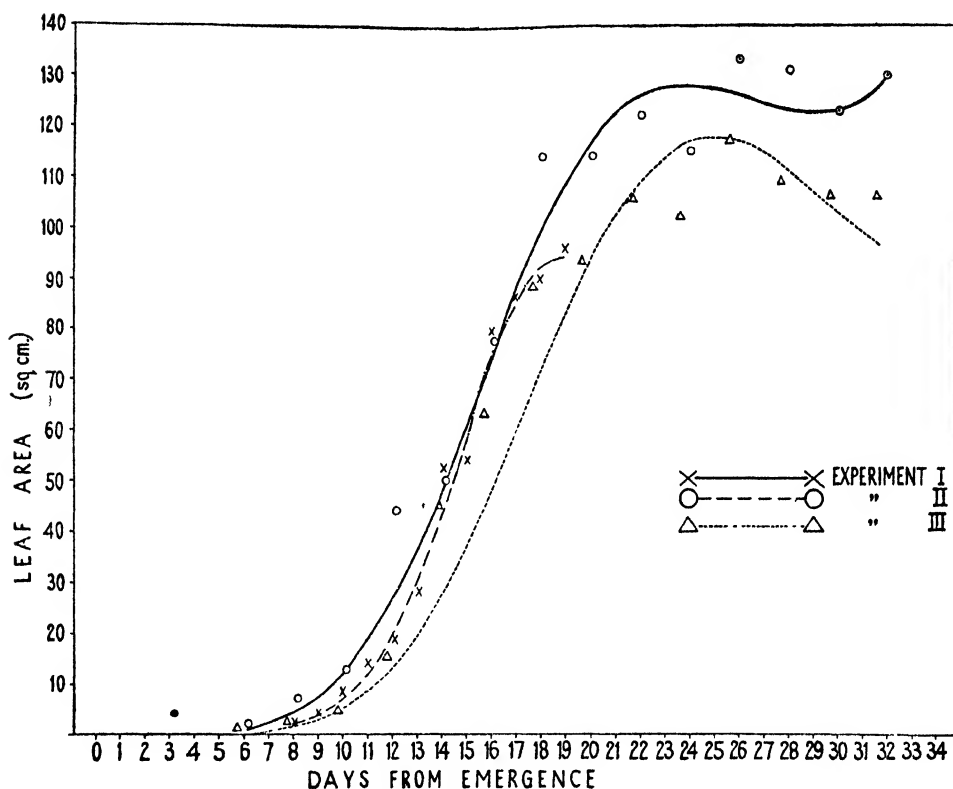


FIG. 4. Course of increase in leaf area of a single plant.

Leaf-area development

The leaf-area data were converted into logarithms before analysis, the standard errors between groups on different occasions proving to be roughly proportional to the means. These logarithms were dealt with in the same way as the data discussed in the previous section, and the results are presented in Table II and Fig. 4. It is clear that leaf expansion may be said to commence at the beginning of the second week after emergence, reaches its maximum rate a week later, and has ceased by the middle of the fourth week. The irregular behaviour after this time in expts. II and III is of doubtful significance.

Water accumulation and distribution

The weight of water in the plants harvested was obtained by difference between the fresh weight and dry weight. The values varied greatly from

TABLE II
Regression on Time of Total Leaf Area

Dependent variable. \log_{10} (leaf area in sq. cm.)	Experiment.	Coefficient of						s^2	s^2_0	s_e	n_0	n
		x^0 ($\times 10^4$)	x^1 ($\times 10^3$)	x^2 ($\times 10^6$)	x^3	x^4	x^5					
\log_{10} (leaf area in sq. cm.)	I	-3.213	+55.18	-14.67	—	—	—	148.4195	0.1303	9	0.1188	99
	II	-2.354	+51.34	-19.55	+24.56	—	—	134.9946	0.3840	10	0.3274	117
	III	-2.879	+51.40	-17.38	+18.93	—	—	222.8425	0.7816	10	0.7267	117

harvest to harvest, and before analysis it was necessary to transform them, as with the leaf-area data, into their logarithms. An analysis of co-variance was performed in order to correct these logarithmic values for the initial seed fresh weight. The correlation and regression coefficients obtained for the

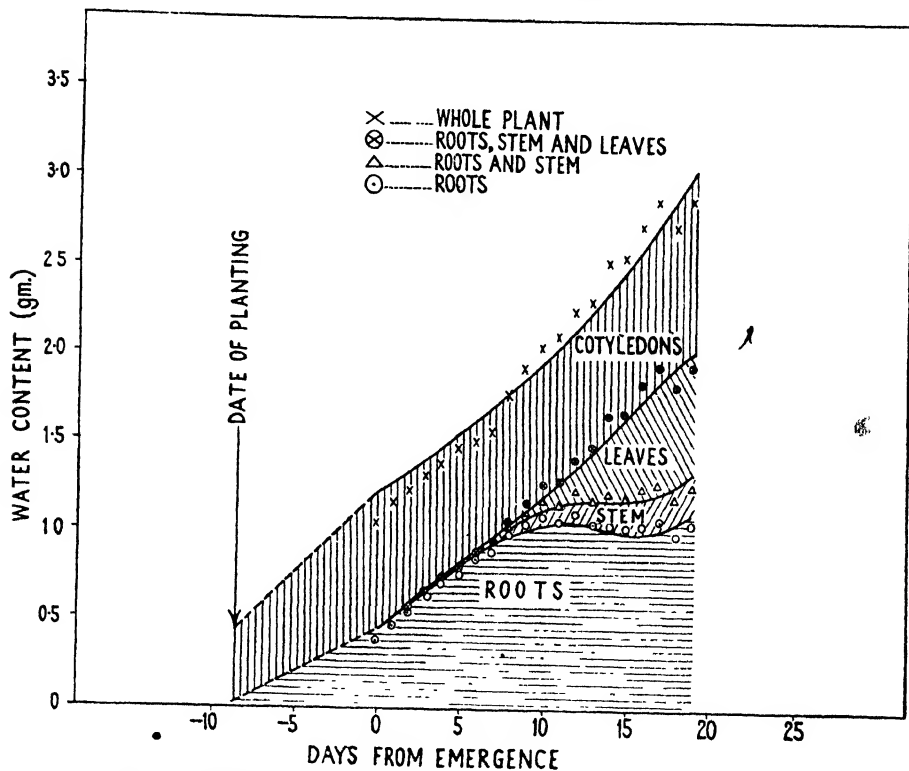


FIG. 5. Changes in distribution of water in a single plant. Experiment I.

interaction term are shown below, the seed fresh weight being expressed in grammes for the group of eight seeds:

	r	b	n
Experiment I . . .	0.633	0.03484	170
Experiment II . . .	0.545	0.02814	197
Experiment III . . .	0.649	0.03545	188

The polynomial regression of the corrected \log_{10} (plant water weight) on time was computed in each experiment, and the results are presented in Table III in the same form as those for plant dry matter in Table I. They are also represented graphically in Figs. 5-7.

As in the dry matter, the regressions recorded in Table III account for a very large proportion of the variance between occasions, but not in most cases for the whole of the excess over the error variance. The plant gains in water content at a rate increasing gradually to about 120 mg. per day during the second week after emergence. The rate of increase then falls off

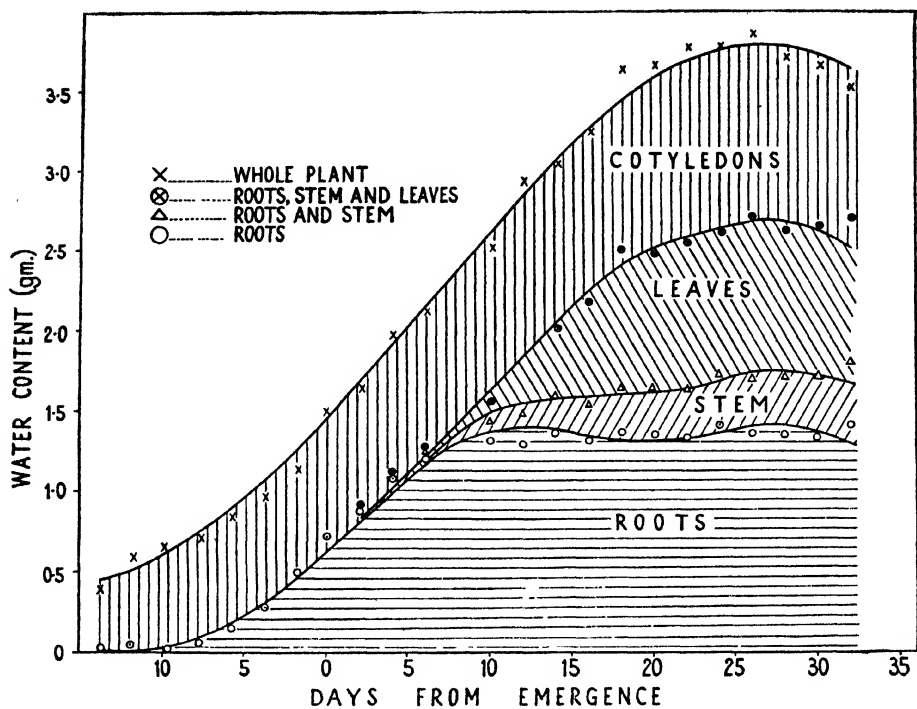


FIG. 6. Changes in distribution of water in a single plant. Experiment II.

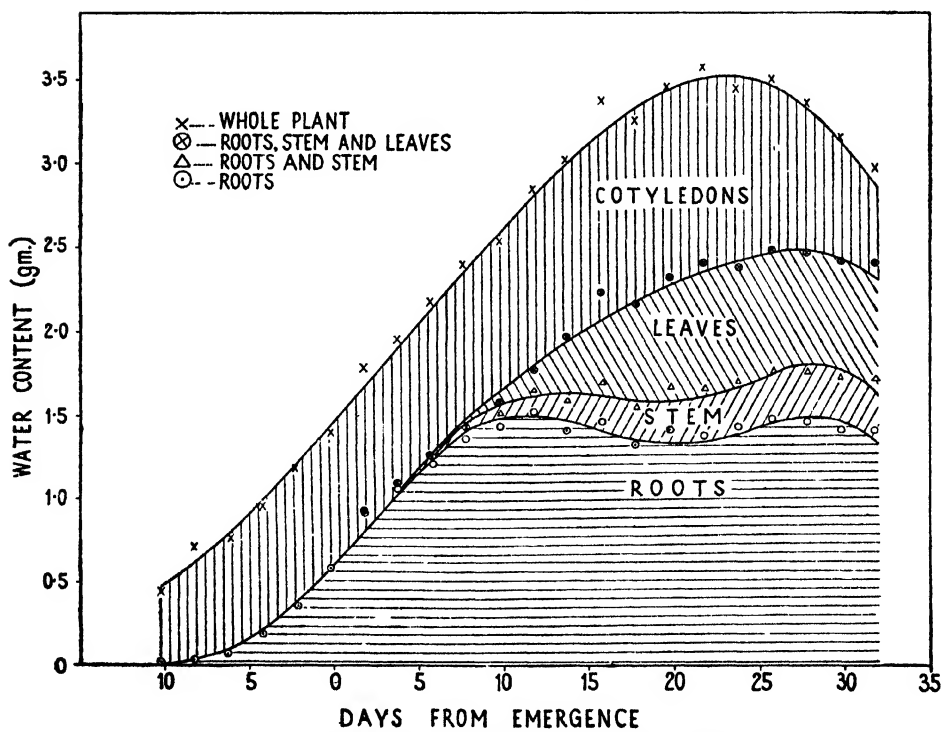


FIG. 7. Changes in distribution of water in a single plant. Experiment III.

TABLE IV
Regression on Time of Water Content as Percentage of Dry Matter

Dependent variable.	Experi- ment.	Coefficient of						s^2	n	s^2	n	s^2	n
		x^0	x ($\times 10^3$)	x^2 ($\times 10^3$)	x^3 ($\times 10^4$)	x^4 ($\times 10^6$)	x^5 ($\times 10^7$)						
Plant	I	2.1854	+6.19	-7.37	+5.53	-14.31	—	7.2436	15	0.0069	171		
	II	2.2174	+3.34	-0.72	—	—	—	59.6735	20	0.0048	198		
	III	2.2610	+3.94	-1.14	+0.06	—	—	42.5158	18	0.0059	189		
Cotyledons	I	2.0190	+3.62	-6.07	+5.81	-15.79	—	7.1903	15	0.0106	171		
	II	1.9940	+1.57	-0.02	+0.39	-1.26	—	35.6470	18	0.0086	198		
	III	2.0700	+1.82	-0.53	+0.78	-2.22	—	25.3334	17	0.0097	189		
Roots	I	2.8910	+1.61	-2.32	+0.74	—	—	0.3087	16	0.0029	171		
	II	2.9099	+1.14	-1.77	+0.73	-1.11	—	14.9991	18	0.0098	198		
	III	2.9400	+0.12	-2.35	+2.51	-10.08	+1.35	6.6652	16	0.0070	189		
Plumule	I	2.6284	—	—	—	—	—	—	5	0.0145	45		
	II	2.6848	—	—	—	—	—	—	1	0.0157	9		
	III	2.5035	+3.50	—	—	—	—	0.1932	0	0.0078	9		
Stem	I	1.5309	+25.52	-16.10	+3.23	—	—	0.4064	8	0.0041	99		
	II	2.4940	+6.05	-3.31	+0.48	—	—	1.0843	9	0.0065	108		
	III	2.4109	+7.03	-3.67	+0.53	—	—	1.6014	10	0.0162	117		
Leaves	I	2.0024	+10.04	-3.47	—	—	—	0.8814	9	0.0084	99		
	II	3.6760	-41.17	+59.12	-36.94	+102.75	-10.53	3.3513	7	0.0092	108		
	III	3.5560	-39.52	+57.11	-35.39	+97.25	-9.85	2.8588	8	0.0111	117		

$\log_{10} (100 \times \text{water weight/dry weight})$

rapidly, and during the fourth week after emergence a maximum is reached and a slow decline commences.

During the first 5 days after planting the major part of the water taken up by the seed remains in the cotyledons, but for the next 2 weeks most of the increase occurs in the roots. The amount of water in the cotyledons continues to increase slowly, however, until about 3 weeks after emergence, when it begins to decline. This decline becomes very rapid in expt. III, over half the maximum water content having been lost by the middle of the fifth week; it is clearly the prelude to abscission. The amount of water in the stem increases most rapidly at about the end of the second week after emergence, but the gain continues at a reduced rate to the end of the experiment, or a little earlier. The water content of the leaves increases very rapidly during the second and third weeks following emergence, forming the main part of the increase in the plant as a whole during the period. Like that of the plant the water content of the leaves reaches a maximum during the fourth week from emergence, decreasing thereafter by some 10 per cent.; this net loss of water is doubtless associated with the 'hardening' of the leaves when expansion is completed, the increase in their area ceasing at about the same time as maximum water content is attained.

Water content as a percentage of dry matter

The weight of water in each organ, and in the plant as a whole, has been expressed as a percentage of the amount of dry matter present, and these data have been analysed in the same way as those described above, using a logarithmic transformation. The polynomial regressions (with variance estimates) are presented in Table IV, and shown graphically in Figs. 8-10.

The percentage water content in the plant as a whole increases from the initial level of about 40 per cent. in the embryo to a maximum of rather over 400 per cent. 3 weeks after emergence, and then decreases slightly as the first flush of leaves hardens. That of the radicle is rather under 200 per cent. at the time of planting and increases very rapidly to a maximum of nearly 900 per cent. shortly after emergence; maturation and secondary thickening of the proximal portion presumably begin at this time, and the percentage water content commences a gradual decline, reaching about 500 per cent. in the fifth week after emergence. The percentage water content of the stems likewise shows an increase, albeit less marked, during the second week after emergence, followed by a decline, and the same is also true of the leaves. It may be noted that the maximum percentage water content of the leaves is reached at a time when expansion in area is still less than half the final figure. The leaves of the flush do not, of course, mature simultaneously, but the attainment of maximum area by successive leaves does not normally spread over more than 4 days. It would appear, therefore, that the fall in percentage water content of the individual leaf must commence before its expansion is complete.

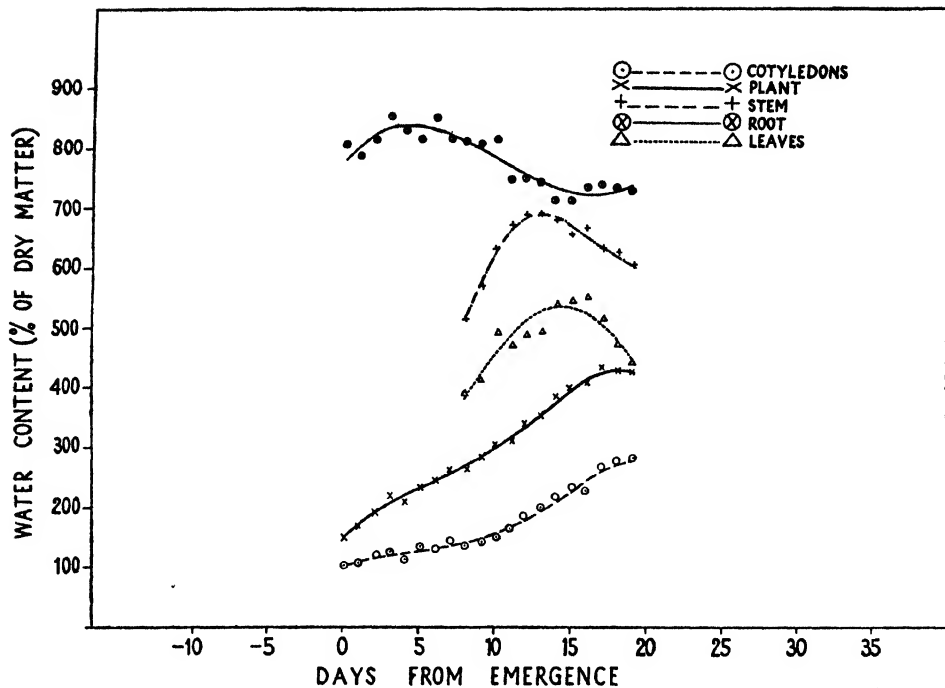


FIG. 8. Water content of the plant and its parts, expressed as percentage of dry matter. Experiment I.

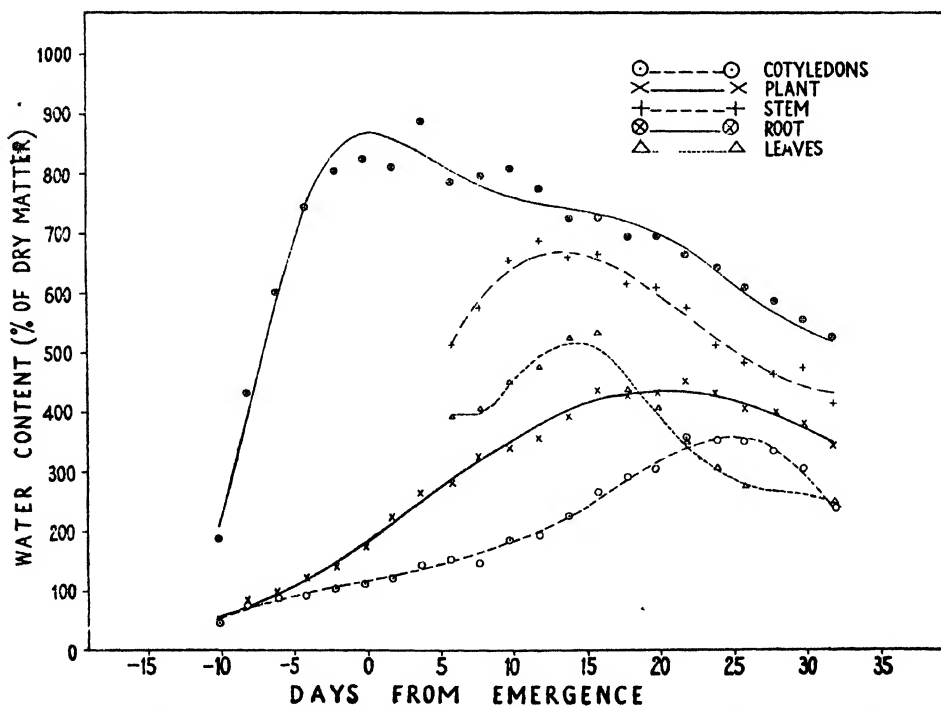


FIG. 9. Water content of the plant and its parts, expressed as percentage of dry matter. Experiment II.

DISCUSSION

As remarked at the outset, very few other quantitative investigations have covered this very early period of development, so that the opportunities for comparison with other species are limited.

Miller's (1910) investigation of germination of sunflower seeds was one of the most exhaustive studies. He harvested the seedlings at five stages,

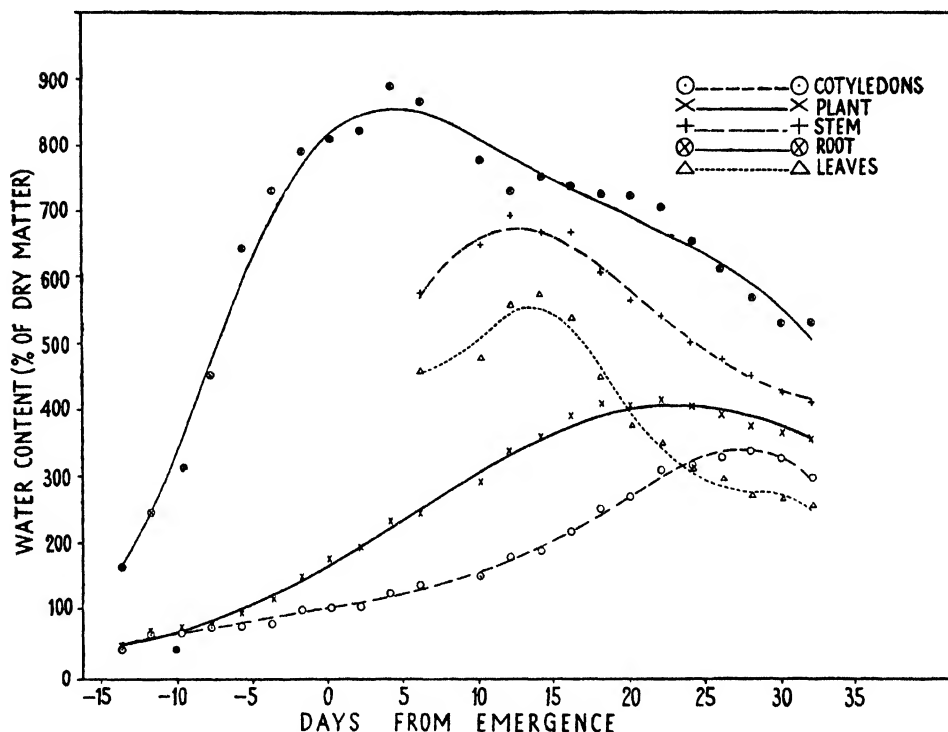


FIG. 10 Water content of the plant and its parts, expressed as percentage of dry matter. Experiment III.

separating cotyledons from roots and hypocotyl, and found that by the time the seedlings emerged their dry weight had diminished by nearly 42 per cent. of the seed weight. The seedling dry matter at this time was divided in the proportion of 72 per cent. in the cotyledons and 28 per cent. in the other organs. Thus at the time of emergence the cotyledons had lost 53 per cent. of their original dry matter, of which 11 per cent. (net) had been translocated to the developing organs and 42 per cent. utilized in respiration; the corresponding figures for cacao are, on average, 17, 9, and 8 per cent. The percentage water content of sunflower cotyledons at the time of emergence was 25 per cent. of the dry matter, that of the other organs 89 per cent.—both figures being very much lower than those for cacao; indeed, the cotyledons of cacao seeds already at planting contain 40 per cent. water.

In certain of Gregory's (1928) experiments with cucumber, dry weights of leaves and shoots (the former including the cotyledons, the latter, apparently, the roots) were recorded at intervals of about 3 days for 19 days following germination. The fresh weight of the seed is given, but unfortunately not the dry weight. In his expt. 3, for example, seeds of 36–7 mg. gave seedlings with a dry weight of 30 mg. on the day following germination, 33 mg. after 4 days, and 37 mg. after a week. In expt. 4 seeds weighing 37–9 mg. gave seedlings with a dry weight of 31.2 mg. at germination, 32.3 mg. the following day, 40.3 mg. after 4 days, and 48.3 mg. after a week. In expt. 5 seeds weighing 36–7 mg. gave seedlings of 28.9 mg. the day after germination, 33.5 mg. after 3 days, and 42.2 mg. after a week. The seeds presumably contained 10 per cent. of water, so the loss in dry matter during the 4 days between planting and emergence can hardly have exceeded 10 per cent. The greater percentage loss in dry matter of cacao seeds during germination is consonant with the longer duration of the process. It is clear, however, that active assimilation commences very promptly in the cucumber seedling, the original dry weight being regained and exceeded after about 4 days, as against 4 weeks for cacao. This difference is presumably due to much greater assimilatory activity of the cotyledons in cucumber, that of the cacao seedling hardly sufficing to maintain the seedling dry weight. Furthermore, the ratio of leaf area to plant dry weight at 3 to 4 days averaged 188 sq. cm. per g. in cucumber, whereas the maximum value observed at any time in the present work on cacao was 135 sq. cm. per g.

Heath's (1937) first determination of the dry weight of cotton seedlings was at 8 days after germination, by which time it was about 75 mg. By extrapolation he concludes that the weight at germination was 43 mg., which would represent a loss of 20 per cent. since planting. 'The growth in weight of the seedling', he says, 'approximates to an exponential curve from the very beginning.' It will be noted that this is far from being true for cacao over the period studied, though the percentage loss before emergence is not dissimilar.

In maize Ashby (1930) found that at 21 days the seedling dry weight was between 69 and 102 per cent. of the seed fresh weight, and was thus presumably almost equal to the seed dry weight. This period of 3 weeks during which the seedling has done no more than maintain its dry weight resembles conditions in cacao more closely than the species considered above; here, however, the similarity ends, for during the following 12 days Ashby's maize seedlings more than doubled their dry weight. At 21 days the mean ratio of leaf area to seedling dry weight was 103 sq. cm. per g., and at 33 days 173 sq. cm. per g. In a subsequent experiment with maize (Ashby, 1932) the seedlings seem to have developed more rapidly; at 7 days their weight was on average 71 per cent. of the initial seed weight, and at 14 days the corresponding value was 125 per cent. Whether these values include the seed residue is not clear; if so. the loss of dry weight during the first week must have been about 15 per cent., a figure very similar to that found for cacao.

Bartel and Martin (1938) also studied the early growth of maize and compared it with that of sorghum and proso (*Panicum miliaceum*); the roots, unfortunately, were not harvested. According to their data the aerial parts of the plant have a dry weight equal to that of the seed in 11–14 days after planting in maize, 8–9 days in sorghum, and 5 days in proso. This point was not reached in the cacao observations, but it would clearly be substantially later than 6 weeks.

Ballard and Petrie's (1936) exhaustive studies of Sudan grass show that 9 days after planting the dry weight of the seedling is already 36 per cent. more than that of the seed, and that at this time the leaves form 23 per cent. of the total plant dry weight. The date of emergence is not stated.

Brown (1946) measured weight changes in barley embryos at intervals of 12 hours during the first 4 days of development. He found that a period of virtually constant dry weight lasting for 48 hours was succeeded by a period of exponential increase, during which the dry weight nearly doubled during each 24 hours. The water content of the embryo increased rapidly from the very beginning of development, reaching 585 per cent. of the dry matter at the end of the fourth day. If the axis of the cacao seedling be taken as the physiological equivalent of the barley embryo, it may be noted that in cacao, too, a similar delay in dry weight increase of from 2 to 4 days is observed (Figs. 2, 3), but subsequent development never approximates to an exponential curve. The rate of increase in water content by the cacao axis is not, however, greatly dissimilar from that in barley embryos; in expt. III (Fig. 10) it increased from 190 to 600 per cent. of the dry matter in 4 days from planting.

Dyer's (1947) experiments were performed with peanuts in darkness. He found that, during the first week after planting, the dry weight of the cotyledons decreased by about 10 per cent., but during the second week by a further 65 per cent. It is clear that the rate of loss from the cotyledons is much greater in peanut than in cacao, but his data do not enable one to find what proportion of the losses from the cotyledons becomes available for the development of the other organs.

To turn to the development of leaf area, the sigmoid curves obtained in these experiments are very different from the exponential curves obtained for the early development of annual plants (e.g. Gregory, 1921). The reason for this, of course, is to be found in the growth habit of cacao, which forms its leaves in 'flushes' rather than in a continuous series. It might well be that, over a prolonged period, leaf-area development in cacao would present a 'stepped' exponential curve.

It is unfortunate that no comparable data for other tree species have come to light, for it is quite possible that the slow tempo of early development in cacao compared with annual crop plants may, in part at least, be a concomitant of the habit of growth. It should also be remembered that the cacao plants in question were growing in a shaded nursery, though evidence at present available indicates that a higher light intensity is not more favourable to the early development of cacao seedlings.

SUMMARY

The course of early growth of the cacao seedling under nursery conditions has been studied by harvests at 1- or 2-day intervals during the first 7 weeks from planting, a total of 5,280 seedlings having been used. On each occasion roots and cotyledons, and in the later harvests stems and leaves also, were harvested separately, and their fresh and dry weights determined, together with leaf area. The results have been analysed statistically, polynomial regression curves for the course of change in each variable being computed.

Loss of dry matter after planting amounts to about 15 per cent. by the time the trend is arrested, 1 week after seedling emergence. The seedling dry weight first exceeds that of the embryo as planted about 4 weeks after emergence. The relative growth rate at this time is about 1 per cent. per day, and the maximum net assimilation rate observed was 0.090 g./sq. dm./week.

Decrease in the dry weight of the cotyledons continues at a fairly constant rate for some 6 weeks, by which time they have lost about 70 per cent. of the original weight of dry matter. They are shed soon afterwards. Increase in root dry weight almost ceases during the third week from emergence, when plumular development is most active, but recommences when expansion of the first flush of leaves comes to an end in the fourth week.

The weight of water in the seedling reaches a maximum during the fourth week after emergence, and thereafter gradually declines to the end of the period studied. For the first 5 days after planting the main increase in water content occurs in the cotyledons, but thereafter the major part of the increase occurs in the root and, later, the plumule. The weight of water in the cotyledons increases until 3 weeks after emergence, then declines until their abscission. The weight of water in the leaves increases rapidly during their development, reaching a maximum during the fourth week from emergence, and declining thereafter during 'hardening'. The decline in the percentage water content of the leaves begins at a time when they are still expanding actively.

These results are compared with quantitative data on the early development of other species; it is pointed out that the relative growth rate and net assimilation rate found for cacao are much lower than data for other species in the literature, and that the period before the cacao seedling regains and surpasses the seed dry weight is exceptionally long.

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Chromosome Numbers in Species of *Neurospora*

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With seven Figures in the Text

INTRODUCTION

THE number of chromosomes in species of *Neurospora* has been variously reported as six and seven. Wilcox (1928) figured approximately six chromosomes in meiosis in the ascospore in *N. sitophila*, but did not determine the number definitely. Colson (1934), working on a strain of *N. tetrasperma* supplied by Dr. B. O. Dodge, found six chromosomes. Recently McClintock (1945) studied the chromosomes of *N. crassa* in some detail, and reported the number as seven. In the same paper she mentions that a strain of *N. tetrasperma* obtained from Dr. Dodge was found to have seven chromosomes. However, Cutter (1946) agrees with Colson in finding six chromosomes in *N. tetrasperma*.

The work here reported was undertaken with a view to determining the chromosome numbers of various stocks of *Neurospora* being used for genetic studies in this laboratory.

MATERIAL

The following five stocks were examined cytologically:

(1) A stock of *N. sitophila* originally isolated from a Chichester timber-yard (Ramsbottom and Stephens, 1935). This was the stock used by Whitehouse (1942) in his linkage studies.

(2) A stock isolated in New Zealand from dead *Nothofagus* leaves by J. H. Warcup. This fungus produces eight-spored asci, but is very difficult to cross with *N. sitophila*, and still more difficult to cross with *N. crassa*. It is morphologically somewhat different from our stocks of either of these species. The frequency of second division segregation shown by the mating-type gene in this stock is in the region of 20 per cent. (7 out of 37 asci dissected), compared with 57.5 per cent. in *N. sitophila* and 14.4 per cent. in *N. crassa* (Whitehouse, 1942). This stock probably deserves to rank as a distinct species.

(3) A stock of *N. tetrasperma* originally isolated from burnt gorse at Woolwich (Ramsbottom and Stephens, 1935).

(4) A stock of *N. tetrasperma* sent from India by E. Hainsworth.

(5) A stock of *N. tetrasperma*, originating from Texas, which was supplied by Dr. B. O. Dodge.

Self-sterile cultures were obtained from these last three stocks by isolating and germinating small ascospores. Using these cultures the three stocks were crossed in all combinations and were found to be fully interfertile, the hybrid asci producing four viable ascospores in every case. This indicates that the chromosome complements of the three stocks must be almost, or quite, identical.

METHODS

The main technique used was a variation of that used by McClintock (1945) and Cutter (1946). These workers squashed out the clusters of asci from young perithecia and stained them directly in a drop of acetocarmine or



FIG. 1.

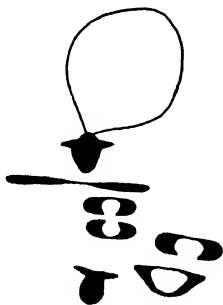


FIG. 2.



FIG. 3.

FIG. 1. *N. sitophila* diakinesis, showing the nucleolar chromosome pair, with an interstitial chiasma, attached terminally to the nucleolus. The largest pair of chromosomes, with two interstitial chiasmata, appear to have median centromeres $\times 1400$.

FIG. 2. New Zealand stock. Metaphase I of meiosis. Seven bivalents, the nucleolar chromosome and one other with interstitial chiasmata. One bivalent with a single terminalized chiasma. $\times 1400$.

FIG. 3. New Zealand stock. Mitotic metaphase in ascospore. A large chromosome with unequal arms, probably attached to the nucleolus by its shorter arm. $\times 2800$.

acetic orcein in the usual way. In the present work this method was not found to give good results. Good staining of the chromosomes was obtained only when the young perithecia had been previously fixed in 1 : 3 acetic-alcohol overnight, followed by mordanting in 4 per cent. iron alum for 4 to 10 minutes, and then washing in water (Godward, 1948). Acetocarmine was found more satisfactory on the whole than acetic orcein. Fixatives containing various proportions of chloroform in addition to acetic acid and alcohol were tried, but were not found to be better than acetic alcohol.

RESULTS

Neurospora sitophila

This species did not prove very amenable to cytological study and rather few satisfactory division figures were found. However, a number of preparations of the first meiotic division were obtained in which seven bivalents could be clearly made out. Not much morphological detail could be seen, but the nucleolus organizer appears to be terminal or sub-terminal on one of

the longer chromosomes (Fig. 1). The largest chromosome pair in the ascus from which Fig. 1 was drawn appears to have more or less median centromeres.

The New Zealand stock

Seven bivalents were also counted in several meiotic figures in this stock (Fig. 2). A good preparation of mitosis in the ascospores was obtained which also showed seven metaphase chromosomes (Fig. 3). From the metaphase shown in Fig. 3 it is possible to get a fairly accurate idea of the relative lengths of the chromosomes and the positions of the centromeres. There are two

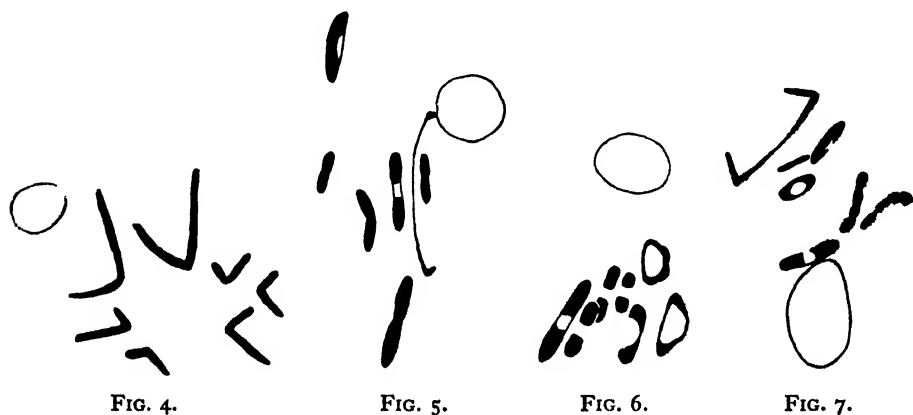


FIG. 4.

FIG. 5.

FIG. 6.

FIG. 7.

FIG. 4. *N. tetrasperma*, Texan stock. Third division in ascus (mitotic metaphase). (In Figs. 3 and 4, a few of the chromosomes which were overlapping have been moved apart slightly to simplify the drawing.) $\times 2800$.

FIG. 5. *N. tetrasperma*, Texan stock. Metaphase I with seven bivalents. One bivalent with a single terminal chiasma. $\times 1400$.

FIG. 6. *N. tetrasperma*, Indian stock. Metaphase I with seven bivalents all with two terminalized chiasmata $\times 1400$.

FIG. 7. *N. tetrasperma*, English stock. Metaphase I. Three bivalents with two terminalized chiasmata, and three with a single terminal chiasma. Of the latter, the two smaller show centromere constrictions, and the large bivalent has approximately median centromeres. The seventh chromosome seems abnormally small for a bivalent and is probably a univalent, the position of the other univalent being uncertain. $\times 1400$.

chromosomes definitely longer than the rest, and one of these has markedly unequal arms and appears to be attached to the nucleolus by its shorter arm. The third-longest chromosome appears to have a sub-terminal centromere. Of the four smaller chromosomes, two have rather unequal and two fairly equal arms.

This stock seems to have much larger nucleoli than the other stocks examined (Figs. 2 and 3).

Neurospora tetrasperma

All three stocks of this species proved to be very favourable cytological material, and in each of the three stocks a number of good first metaphases of meiosis were found. Throughout the work chromosome counts were found

to be easiest at this stage. All these figures showed seven bivalents. In some of the preparations two of the chromosome pairs were seen to be considerably larger than most of the others. One of these is frequently found attached to or associated with the nucleolus, and presumably carries the nucleolar organizer (Fig. 7). The ascus from which Fig. 5 was drawn shows that the large chromosome not carrying the nucleolar organizer has a more or less median centromere. Two of the smaller bivalents in the preparation showed more or less median constrictions which were interpreted as centromere constrictions.

A few good preparations of the third (mitotic) ascus division and of mitosis in the ascospore were obtained. From these it was possible to get a more accurate idea of the relative lengths of the chromosomes and the positions of the centromeres (Fig. 5). Two of the chromosomes are much longer than the rest, one having a sub-median centromere, and the other rather unequal arms. Of the other chromosomes, one has markedly unequal arms and four have more or less median centromeres.

Chiasma frequency in the different stocks

In most of the metaphase I and diakinesis figures it was possible to obtain an approximate count of the number of chiasmata, and it was also often possible to classify the chiasmata as terminal or interstitial. In only a few cases, however, could all seven bivalents be analysed in this way, so reliable figures are hard to obtain. Some differences between the stocks did, however, emerge.

Owing to the high frequency of terminalization, an accurate count of chiasma frequency is impossible at metaphase I, and rather few diakinesis stages were found. However, bivalents having only a single chiasma were generally distinguishable. The number of these observed in the different stocks, and the resulting minimum chiasma frequencies, are given in the following table:

	No. of bivalents observed.	No. with one chiasma.	Minimum chiasma frequency.
<i>N. sitophila</i>	56	13	1.77
N.Z. stock	35	3	1.91
<i>N. tetrasperma</i> , Indian	42	0	2.0
„ English	56	10	1.82
„ Texan	308	23	1.92

N. sitophila and the English *N. tetrasperma* seem to have a relatively low and the Indian *N. tetrasperma* a relatively high chiasma frequency. All cultures were raised at 25°C.

There also seemed to be differences in the frequency of chiasma terminalization in the different stocks. Out of 58 metaphase I's examined in the three *N. tetrasperma* stocks, only four certainly non-terminalized chiasmata were found, and these were all in the Texan stock. In *N. sitophila* four non-terminalized chiasmata were seen in eight metaphase I's, and eight or nine were found in five metaphase I's in the New Zealand stock. It seems that

the frequency of terminalization of chiasmata is higher in *N. tetrasperma* than in the eight-spored species examined.

DISCUSSION

It seems clear that all the stocks examined have a haploid complement of seven chromosomes. The fact that three stocks of *N. tetrasperma* from such diverse localities as England, India, and Texas all have seven chromosomes makes it seem likely that this number is general for the species. If six-chromosome strains did occur, they would be expected to be rather sterile when crossed with seven-chromosome strains. So far as the writer is aware, no such sterility barriers within the species have been reported.

McClintock reported that the nucleolar chromosome of *N. crassa* has a sub-terminal nucleolar organizer carried on the shorter of the chromosome arms, and was the second-largest chromosome. This also seems to be the case in the New Zealand stock, and probably also in *N. sitophila*. The largest chromosome in *N. crassa* has a sub-median centromere, according to McClintock, and this may be homologous with the large chromosome with more or less median centromere found in all three species studied by the writer. *N. tetrasperma* also agrees with *N. crassa* in having the nucleolar organizer situated on one of the two largest chromosomes, probably one with unequal arms. In *N. tetrasperma* and the New Zealand stock, where some idea of the morphology of all the chromosomes was obtained, a fairly good correspondence was found with the chromosomes of *N. crassa* as reported by McClintock (1945). The third-largest chromosome in the New Zealand stock (Fig. 3) appears to have a sub-terminal centromere, not a sub-median one as tentatively reported for *N. crassa*. With such small chromosomes, however, too much emphasis cannot be given to conclusions based on a single division figure.

So far as these results go they are consistent with the view that the chromosomes of *N. crassa*, *N. sitophila*, *N. tetrasperma*, and the New Zealand stock are rather similar morphologically. The haploid number of seven is probably general for the genus.

SUMMARY

The chromosomes of three stocks of *Neurospora tetrasperma*, a stock of *N. sitophila*, and a probable new species from New Zealand have been studied.

The haploid number in all five stocks is seven.

No marked morphological differences between the chromosome sets of the different species were observed, although there are indications that terminalization of chiasmata is more frequent in *N. tetrasperma*.

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I am indebted to Dr. D. G. Catcheside for much helpful criticism and advice.

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The Salt Relations of Plant Tissues

IV. Some Observations on the Effect of the Preparation of Storage Tissue on its Subsequent Absorption of Manganese Chloride

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With ten Figures in the Text

INTRODUCTION

THE object of this investigation was to ascertain how far the absorption of dilute manganese chloride by storage tissue is dependent on, and may be modified by, the manner in which the tissue is treated before bringing it into contact with the salt solution.

Discs of storage tissue as plant material for experiments in salt absorption have been widely used by different investigators during the last 40 or so years and are still considered a most suitable subject for these investigations, especially when it is desirable to connect salt-intake data with respiratory measurements, or with biochemical analyses of metabolites which may be undergoing changes during this process. However, although the use of living cells in the form of discs of constant size are very convenient for obtaining quantitative data, there is one drawback inherent in the method, namely the necessity for cleaning and subsequent handling of the discs after cutting in preparation* for the experiments. It has been the custom of many workers to wash the discs for varying periods in tap- or distilled water, in order to remove electrolytes and other contents from cells injured by disc preparation. Thus Stiles and Jorgensen washed potato discs for about an hour, Robertson using carrots a few hours, while Asprey prepared potato tissue by washing it for some 16 or 20 hours in tap-water. In some cases the tissue has been previously aerated during preliminary tap-water preparation (Robertson, 1941; Stiles and Dent, 1946), although there is no mention of aeration by many other workers. That previous washing of tissue in tap-water does affect the subsequent uptake of salts has been shown by Asprey (1933), Steward and Berry (1943), and Stiles and Dent (1946). Asprey subjected potato and artichoke to prolonged washing for periods varying from 1 day to 191 hours and showed that previous differential washing did enhance the uptake of both ions of ammonium chloride by potato tissue, but no such effect was observable in the case of artichoke tuber. Steward and Berry (1943) suggest that the effect of previous washing of potato tissue is well known and that the effect of protracted washing of tissue may be attributed to temperature, salt content, and oxygen concentration. They state they used arbitrary standardization of

the duration of washing during any series of experiments. Stiles and Dent demonstrated that prolonged washing in aerated tap-water of carrot and red beet influenced and enhanced the amount of both cation and anion subsequently absorbed from dilute manganese chloride solutions by these tissues, the results being more marked in the case of beet. Using the polarographic method of ion estimation, they showed that protracted washing not only increases the amounts of respective ions absorbed in unit time but greatly influences the course of ion intake. The second phase of long-continued absorption is rapidly accelerated, while the lag or induction period, shown by cation and anion absorption curves after immersion of the tissue in the salt solution, is lessened or eliminated by prolonged washing.

In the experiments described in the present investigation, storage tissue was deliberately subjected to different conditions in order to ascertain how these various treatments would affect subsequent salt intake. Treatments included washing in tap-water and leaching in distilled water, both in the presence or absence of air. The direct effect of air, oxygen, and nitrogen and finally the effect of previous temperature treatments on subsequent intake of manganese chloride were examined.

EXPERIMENTAL METHODS

Three tissues were employed: red beetroot (Suttons' Crimson Globe), artichoke (unknown variety), and parsnip (Simpsons' Student), all of which were grown in the University of Birmingham grounds. The experimental procedure after pretreatment was identical with that used by Stiles and Dent (1946). The storage tissue was employed in the form of discs 2 cm. in diameter and 1 mm. thickness, used in replicate batches of 20 in 200 ml. of 0.001 M. manganese chloride contained in 420-ml. bottles whose stoppers contained a 1-cm. diameter hole, and which were shaken continuously in a bath maintained at 25° C. by means of the Sun-Vic thermostat. At intervals during the experiments 10 ml. of the external solution were removed along with one disc and analysed for manganese and chloride respectively, using a Cambridge Instrument Company's polarograph fitted with a galvanometer damping device. This amount of solution is more than adequate for polarographic determinations since only 2 ml. for manganese and a similar amount for chloride is needed, which means that duplicate determinations may be made of each replicate solution. The results presented are the average of such determinations. The accuracy and advantages of the polarographic method have been amply outlined in the previous paper in this series. The advantages and accuracy, when a galvanometer damping system is inserted in the polarographic circuit, are outlined by Lingane and Kerlinger (1940). The advisability of analysing the external solution, where determinations may run into thousands as in the present investigation, is also dealt with in detail by Stiles and Skelding (1940). The figures submitted, as data obtained, are given as absorption as a percentage of the concentration of the original external solution, which was 0.001 M. manganese chloride throughout.

EXPERIMENTAL RESULTS

I. *The Effect of Washing in Aerated Running Tap-water on the Absorption of Manganese Chloride by Storage Tissue*(a) *Parsnips* (1946)

Experiment 55: Discs were cut on February 25 and washed in aerated running tap-water for 24 and 168.5 hours respectively. After these intervals the discs were removed, quickly blotted, and transferred to 0.001 M. manganese chloride at 25° C. Samples were withdrawn as outlined above at suitable intervals, and the concentration of manganese and chloride determined polarographically.

The results are shown in figures in Table I and graphically in Fig. 1.

TABLE I

The Effect of the Duration of Washing on the Absorption of Manganese Chloride by Parsnip Tissue. Expt. 55

Hours of washing.	Hours immersed in 0.001 M. MnCl_2 .	% absorption of Mn.	% absorption of Cl.
24	5	38.85	—
	25	46.42	5.34
	48	55.46	9.37
	72	60.75	21.88
	95.8	68.88	45.80
	144.8	79.37	69.78
	168	—	74.26
	193	—	77.58
	216	—	83.48
168.5	5	45.05	4.51
	24	55.75	15.49
	48.1	67.37	43.47
	72.3	80.94	71.06
	96	89.77	86.18

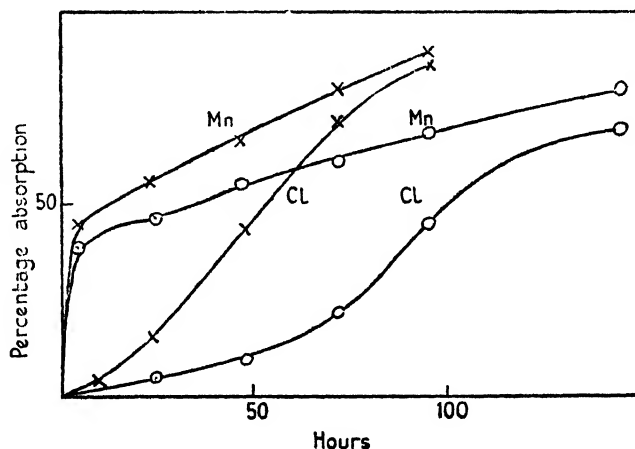


FIG. 1. The course of absorption by parsnip tissue of the ions of manganese chloride from a solution of an initial concentration of 0.001 M. ○, after previous washing in aerated tap-water for 24 hours; X, after washing for 168.5 hours.

The graphs illustrate the inequality of absorption of cation and anion in the early stages, but the rates become comparable towards the end of the experiment. With longer washing the lag period is almost eliminated in the case of cation, while both ions are absorbed more rapidly.

These results are in complete agreement with those obtained by Stiles and Dent (1946) with carrot and beet. Here again the appearance of the second phase is accelerated by washing.

(b) *Artichoke*

Experiments with artichoke were conducted on January 29 and March 11 respectively. The results are given in Table II and Fig. 2.

TABLE II

The Effect of the Duration of Washing on the Absorption of Manganese Chloride by Artichoke Tissue

Hours of washing.	Hours of immersion in 0.001 M. $MnCl_2$.	% absorption of Mn.	% absorption of Cl.
<i>Experiment 54</i>			
24	5	20.84	6.19
	24	26.70	13.85
	48	32.93	23.58
	71	34.21	30.29
	120	40.08	45.14
	143.3	44.51	—
168	5	26.79	0.49
	24	30.53	9.19
	48	31.84	17.44
	71	32.11	23.51
	94.1	33.70	25.84
	145.2	34.21	29.68
<i>Experiment 56</i>			
24	5	20.66	4.5
	24	28.53	15.74
	48	34.84	30.12
	72	37.50	41.06
	92	42.07	47.62
	144	50.37	54.75
168	5.1	24.81	4.5
	24	28.72	9.3
	48	32.83	24.9
	72	34.37	34.37
	98	36.8	45.58
	168	44.7	54.75

It will be observed that in neither experiment does prolonged washing in tap-water result in an increased rate of absorption. In expt. 54 there is a slightly enhanced absorption of both ions during the first 48 hours, after which tissue which has been washed for the shorter period absorbs more ions than that which has been subjected to more lengthy washing. These observa-

tions are in accordance with results given by Asprey, who found that the intake of both ions of ammonium chloride by artichoke was slightly retarded when this tissue was washed in tap-water for periods varying from 24 to 191 hours.

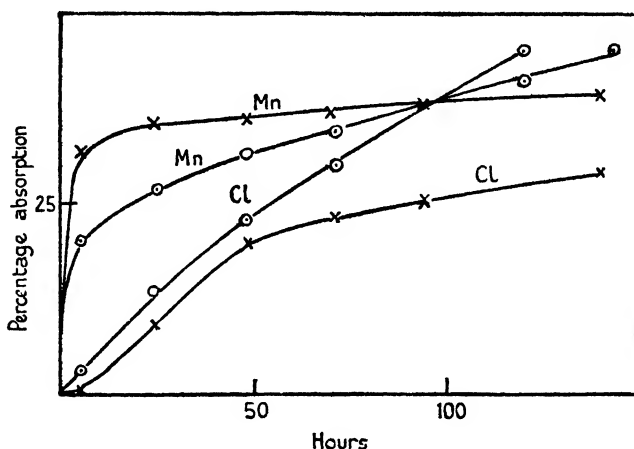


FIG. 2. The course of absorption by artichoke tissue of the ions of manganese chloride from a solution of an initial concentration of 0.001 M. O, after previous washing in aerated tap-water for 24 hours; X, after washing for 168 hours.

II. The Effect of Washing in Tap-water from which Air is excluded, on the Subsequent Absorption of Manganese Chloride by Parsnip Tissue

Results of the previous experiments indicate that parsnip tissue behaves like potato tuber (Asprey), carrot, and beet (Stiles and Dent), with regard to its prolonged washing, in that such treatment induces these tissues to absorb certain ions more rapidly. One factor responsible for this extra-induced absorption may be the effect of air in the water. This fact has been previously suggested by Steward and Berry (1943) and Stiles and Dent (1946). As parsnip tissue appears to be particularly sensitive to prolonged washing, experiments were devised in which air was excluded from the tap-water while this tissue was being washed. Discs were prepared from a number of parsnip roots on May 13. They were thoroughly mixed and divided into two equal batches. Each batch was placed individually into a special type of washing vessel, which consisted of a long, closed tube of about a litre in capacity, with two side tubes through which water could be admitted and allowed to flow away. Into the lower end of the vessel was a sealed fine sinter plate through which air or nitrogen could be admitted. The flow of tap-water through the apparatus was controlled at 45 litres per hour. At intervals of 24, 72, and 144 hours respectively replicate samples of twenty discs were removed, quickly dried and placed in 200 ml. of 0.001 M. MnCl_2 as before, and the course of absorption followed in the usual way.

The results are given in figures in Table III and by graphs in Figs. 3 and 4.

TABLE III

The Effect of Washing of Parsnip Tissue on its Subsequent Absorption of Mn and Cl Ions from 0.001 M. $MnCl_2$ when Aeration is promoted and excluded

Washing in hours.	Hours immersed in 0.001 M. $MnCl_2$.	% absorption of Mn.		% absorption of Cl.	
		Aerated.	Air excluded.	Aerated.	Air excluded.
24	5.1	37.8	39.6	2.8	2.8
	24	43.2	44.0	14.3	11.1
	48	53.2	50.1	28.6	20.8
	72	61.0	—	38.2	32.8
	106	70.6	69.8	57.7	53.7
	120	73.9	71.5	61.2	56.8
	144	78.4	76.5	68.3	64.9
	168	82.6	78.7	74.0	67.1
72	5.0	38.7	40.0	6.4	—
	24	51.5	48.5	19.4	15.2
	58.2	70.7	63.8	45.2	28.8
	72	76.1	66.8	51.6	40.0
	96	81.8	74.8	69.0	55.2
	120	86.1	78.6	76.1	61.0
	144	88.3	82.8	81.6	66.2
	168	89.7	85.7	88.9	74.2
144	5	40.2	39.9	11.4	5.4
	24	53.9	48.6	28.4	20.9
	48	70.4	59.4	46.4	37.5
	72	81.3	—	64.3	46.2
	96	90.7	75.9	87.7	58.9
	120	98.6	79.6	—	67.2
	192	—	89.3	—	86.3

Two important facts emerge from these results, the first being that exclusion of air from parsnip discs in running tap-water retards the subsequent absorption of both ions by this tissue as compared with salt intake of the aerated tissue. The pairs of graphs *a*, *b*, and *c* of Figs. 3 and 4 illustrate this fact and it will be observed that the disparity between each pair increases with prolonged washing. It has been shown that prolonged washing of parsnip with aerated tap-water furthers subsequent salt intake. This is well shown in the air curves in Figs. 3 and 4, but if the nitrogen curves for each ion are also compared (and also the numbers in columns 4 and 6, Table III) it is at once seen that prolonged washing still enhances absorption when air is excluded, but this increased absorption at each stage of washing is less when air is excluded than when aeration is promoted.

Considering now the cation absorption graphs of Fig. 3, curves *c* show the typical two-phase absorption of manganese, followed by a lag and then a steady rate of absorption characterizing the second phase. Comparing curves *a*, *b*, and *c* respectively, it will be at once observed that the second phase is accelerated with prolonged washing whether the discs are in nitrogen or air during washing. It is evident that the presence of air in running tap-water is

one factor responsible for increasing subsequent uptake. But it is not the only factor, since when air is excluded prolonged washing is still effective in favouring salt uptake.

Another factor connected with washing in tap-water, and which has been mentioned in the past as a possibility in exerting an effect on tissue, is the

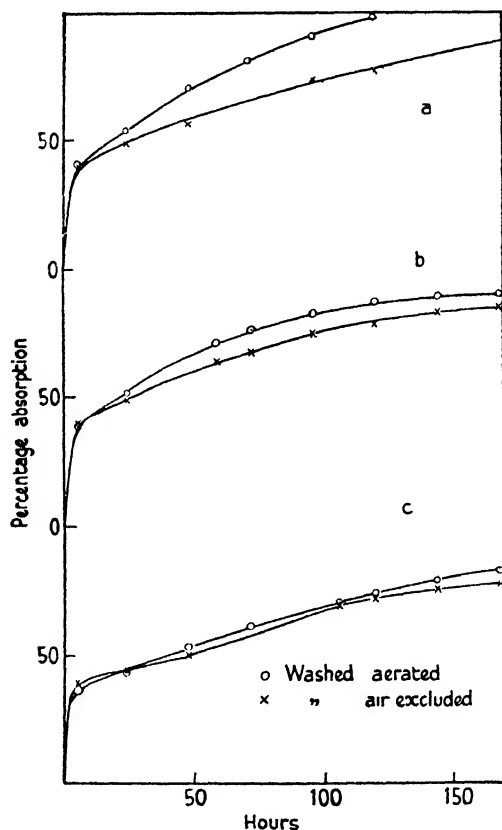


FIG. 3. The course of absorption by parsnip tissue of manganese ions from a solution of manganese chloride of an initial concentration of 0.001 M. *a*, after previous washing in tap-water for 144 hours; *b*, after previous washing for 72 hours; *c*, after previous washing for 24 hours

leaching effect during washing (Asprey, 1937; Steward and Berry, 1943). In this respect Stiles has shown that if storage tissue such as carrot, artichoke, turnip, parsnip, potato, or red beet be kept shaken in contact with distilled water, there is first a loss of electrolytes by the cells followed by an absorption. The most critical experiments were performed with beet shaken in the form of discs in distilled water at 20° C., when it was noted that there was a rapid diffusion out of electrolyte from the tissue during the first day, followed by a gradual reabsorption over a period of some days. It occurred to the writer that it might be of interest to examine the intake of manganese chloride by

tissue thus treated, and to compare the absorption by discs which had lost their electrolytes with those which had reabsorbed them. Experiments were thus conducted accordingly.

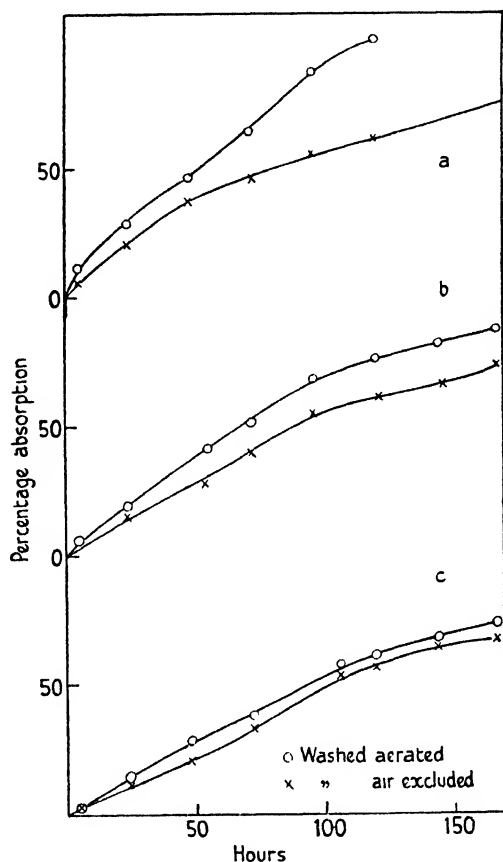


FIG. 4. The course of absorption by parsnip tissue of the chloride ions from a solution of manganese chloride of an initial concentration of 0.001 M. *a*, after previous washing in tap-water for 144 hours; *b*, after previous washing for 72 hours; *c*, after previous washing for 24 hours.

III. The Effect of Previous Immersion in Distilled Water of Red Beet on its Subsequent Absorption of Manganese Chloride

Discs were cut from one red beetroot on September 23. They were rinsed in seven changes of distilled water of 10 minutes each to remove the contents of dead cells. No red pigment was visible in the last washing after this treatment. The discs were quickly dried between blotting-paper and divided into three groups which were treated accordingly.

Group A. Placed in 0.001 M. manganese chloride and the absorption of cation and anion measured polarographically.

Group B. Placed in distilled water in the shaker, and the specific conductivity of the external solution measured at intervals in order to follow the

course of diffusion of electrolytes out of the tissue. When this diffusion had reached its maximum (after 25.9 hours) the discs were removed from the distilled water and rinsed quickly in distilled water to remove electrolytes of the external liquid which were adhering to the disc surface.

They were then quickly dried with blotting-paper and treated as group A.

Group C. Were treated similarly to group B but kept in distilled water until practically all the electrolytes lost by the discs were reabsorbed. In this experiment this took place after 167.9 hours. The tissue was then removed, placed in 0.001 M. MnCl_2 and the absorption of the salt measured. The results are given in Table IV.

TABLE IV

The Effect of Immersion of Red Beet Tissue in Distilled Water on its Subsequent Absorption of Mn and Cl Ions from 0.001 M. MnCl_2 (Expt. 71)

Hours immersed in distilled water in shaker.	Hours immersed in 0.001 M. MnCl_2 .	% absorption of Mn.	% absorption of Cl.
0.0	6.7	13.3	-7.3
	24.5	19.5	-4.9
	48.0	20.9	-2.4
	72.0	23.5	2.4
	94.7	25.0	8.6
	144.0	38.8	35.3
	168.0	47.2	43.9
25.9	4.3	16.6	0.7
	24.0	22.2	7.5
	49.2	23.8	13.3
	68.7	30.0	24.1
	120.0	44.4	29.2
	144.0	49.6	46.8
	168.0	69.4	56.2
167.9	5.0	19.4	1.2
	24.0	41.7	20.0
	48.6	44.4	33.4
	72.0	56.1	54.3
	96.0	66.4	67.8
	144.5	86.1	90.0
	168.0	94.4	96.5

In the second experiment, which was conducted on October 29, the maximum diffusion of electrolytes out of the tissue took place after 28 hours, while all electrolytes had been reabsorbed after the tissue had been immersed in distilled water for 70 hours; the results are recorded in figures in Table V and graphically in Fig. 5.

A consideration of the curves derived from these data as shown in Fig. 5 demonstrates that tissue from which electrolytes have been leached by distilled water does undoubtedly absorb more of both ions than that which has not been so treated, but when such tissue has been allowed to reabsorb these electrolytes, its ability to absorb manganese chloride is still further accelerated.

TABLE V

The Effect of Immersion of Red Beet Tissue in Distilled Water on its Subsequent Absorption of Mn and Cl Ions from 0.001 M. MnCl_2 (Expt. 72)

Hours immersed in distilled water in shaker.	Hours immersed in 0.001 M. MnCl_2 .	% absorption of Mn.	% absorption of Cl.
0.0	5.0	19.1	2.58
	24.2	21.9	3.93
	48.7	27.3	15.76
	70.5	35.86	32.84
	95.0	45.72	50.82
	119.2	58.55	63.40
	144.3	76.35	78.68
28.0	4.1	20.2	2.58
	23.1	23.9	7.90
	42.9	34.6	28.90
	67.0	45.18	50.77
	96.2	58.1	64.24
	120.2	84.55	84.20
70.0	25.2	40.48	33.28
	48.5	52.39	49.36
	77.2	71.19	67.48
	96.5	88.21	88.27
	127.5	96.72	—

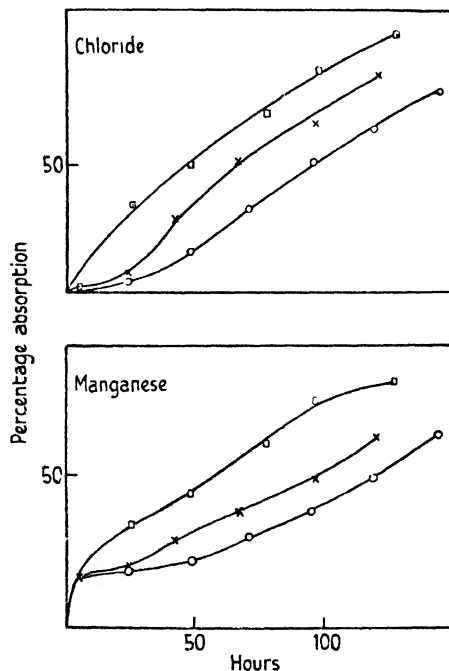


FIG. 5. The course of absorption by red beetroot tissue of the ions of manganese chloride from a solution of an initial concentration of 0.001 M. After previous immersion in distilled water at 25° C. for ○, 0.0 hours; ×, 28.0 hours; □, 70.0 hours.

The curves are reminiscent of those obtained by Stiles and Dent (1946) during prolonged washing of beetroot in tap-water and by the writer for parsnip tissue similarly treated.

If the loss of solutes by washing or leaching was a predominant factor responsible for the tissue absorbing more salt, which extra absorption is, in fact, seen with the discs treated as in group B, one would have expected the course of absorption from discs which had lost their electrolytes to have been in excess of those which had regained them. While it is quite possible that this effect is operating, it is evident that some other more potent factor is associated with the process. In all probability this factor is the contact of the discs with dissolved air in the distilled water which is constantly being replaced during the repeated shaking of tissue in bottles open to the external atmosphere. If, indeed, the removal or addition of electrolytes to the tissue does respectively accelerate and retard its ability to absorb manganese chloride, such effects are probably masked by the effect of the continuous contact with air.

That contact of storage tissue with air and oxygen does significantly affect its subsequent absorption of manganese chloride is shown in the next section.

IV. *The Effect of Previous Contact of Red Beet Tissue with Air, Nitrogen, and Oxygen on its Subsequent Absorption of 0.001 M. Manganese Chloride*

The experiments described in this section are of two kinds, involving slightly different techniques in exposing the tissue to the gases under consideration before measuring the effect on absorption. In the first method to be described the discs were placed in distilled water in a gas-absorption jar with a sinter plate sealed into its base. Moist air, nitrogen, and oxygen respectively were passed for 24 hours into the gas-absorption jars which were kept in a constant temperature bath at 25° C. During this time it has previously been shown by Stiles and confirmed by the writer that the maximum amount of diffusible electrolytes leave the tissue and pass into the external solution.

The experiments, while illustrating the effect of certain gases on tissue behaviour, are closely linked with those of the preceding section and serve to illustrate how the removal of electrolytes from storage tissue under conditions where air is excluded may affect its subsequent absorption of solutes.

In the second series of experiments the discs were placed a centimetre apart on glass racks, the whole being kept in a closed tubular plant chamber fitted with a gas entry and exit tube. Distilled water was admitted into the chamber in such quantity that a small part of the diameter of each disc was always in contact with water. Under these conditions the discs remained moistened if the gas was first passed through a sintered gas-absorption jar containing sterile distilled water. All parts were sterilized before use and the whole apparatus held in a constant temperature bath at 25° C.

In the first method discs from one beetroot were cut on November 12 and divided into four replicate groups. The first group was dried, weighed, and transferred to 0.001 M. manganese chloride at 25° C. The other three groups were subjected to a steady stream of nitrogen, oxygen, and air respectively, in

gas-absorption jars in distilled water at 25° C. The gas treatment was continued for 24 hours, after which the discs were removed and transferred to 0.001 M. manganese chloride at 25° C. The external solution was analysed in all cases polarographically. The results are shown in Table VI and Fig. 6.

TABLE VI

The Course of Absorption of Ions from 0.001 M. Manganese Chloride by Discs which have been previously treated with Nitrogen, Oxygen, and Air in Distilled Water

Percentage Absorption of Manganese.				
Time in hours.	Untreated.	Nitrogen-treated.	Oxygen-treated.	Air-treated.
5	18.32	18.7	21.86	19.07
24	19.49	25.9	27.06	22.82
48	21.32	27.91	40.69	32.92
72	27.5	51.72	62.16	62.60
120	58.5	76.21	78.4	79.59
144	71.71	77.38	82.1	82.20
168	75.21	80.62	88.22	88.2
192	77.4	84.26	100.0	100.0
Percentage Absorption of Chloride.				
5	—1.99	—0.23	5.31	2.34
24	0.49	2.45	15.96	9.3
48	1.49	16.16	35.40	27.33
72	20.39	39.60	59.24	60.50
96	40.79	53.5	—	70.4
120	51.8	60.12	72.62	74.63
144	58.9	62.68	76.42	81.18
168	64.2	72.68	81.0	—
192	73.18	79.06	83.79	—

Examination of the manganese absorption curves in Fig. 6 elucidates the following points. All graphs show the typical two-phase cation absorption, initial rapid intake of the ion followed by a slowing down of the absorption rate and a second steady phase of intake. But in the case of gas-pretreated tissue the arrival of the second phase of absorption is greatly accelerated. This effect is even quite marked where nitrogen is used and the greatest with air or oxygen. The enhanced absorption due to oxygen as compared with air is insignificant and observable only in the initial absorption. The course of chloride intake is similarly influenced. The removal of electrolytes by distilled water when air is excluded still results in a marked increase in the subsequent intake of both ions.

In the previous experiments the action of gases on tissue during leaching has been studied, but it is of interest to investigate the effect of direct action of air, nitrogen, and oxygen on the tissue when electrolytes are not being lost. The method of analysing this effect has already been outlined.

Discs for expt. 57 were cut on May 9 and were divided into two batches. The first batch was placed directly into 0.001 M. manganese chloride at 25° C., the second batch was subjected to a steady stream of air for 24 hours,

as already described in a previous paragraph. They were then removed and placed in 0.001 M. manganese chloride at 25° C. Samples of the external

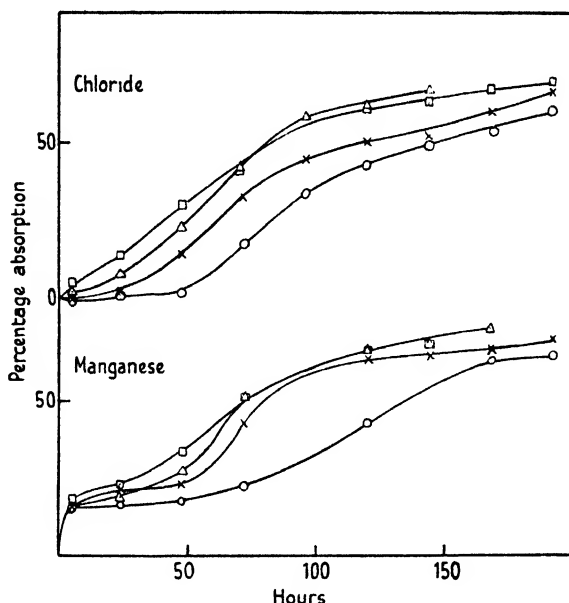


FIG. 6. The course of absorption by red beetroot tissue after different gas pre-treatment in distilled water of the ions of manganese chloride from a solution of an initial concentration of 0.001 M. ○, untreated tissue; X, nitrogen-treated; □, oxygen-treated; △, air-treated.

solutions in both cases were removed at intervals over the course of a week and analysed polarographically. The results are shown by the data of Table VII and graphically in Fig. 7.

TABLE VII

The Course of Absorption of Ions from 0.001 M. Manganese Chloride by Red Beet which has been previously treated with Air (Expt. 57)

Time in hours.	Percentage absorption.			
	Untreated tissue.		Air-treated tissue.	
	Mn.	Cl.	Mn.	Cl.
5	23.39	—	26.61	5.84
24	28.53	4.37	30.63	10.42
48	29.63	11.25	37.40	26.64
72	34.02	27.2	45.78	40.0
96	—	—	60.30	58.36
96.1	46.05	40.60	—	—
120	58.52	49.80	72.94	67.90
144	73.64	66.70	—	—
144.1	—	—	82.30	78.58
168	82.18	76.43	87.0	84.82
192	85.24	80.88	92.42	86.88

A comparison of Figs. 6 and 7 clearly demonstrates that the enhanced absorption attributable to previous contact with air is greater in the case of

tissue which is being leached during the process of aeration than is the case where the gas is brought into contact with the discs which retain their electrolytes.

Experiments with nitrogen, which are illustrated by Table VIII and Fig. 8, also demonstrate that the effect of this gas alone is less than when it is applied in conjunction with distilled water.

TABLE VIII

The Course of Absorption of Ions from 0.001 M. Manganese Chloride by Red Beet which has been previously treated with Nitrogen

Time in hours.	Percentage absorption.			
	Untreated tissue.		Nitrogen-treated tissue.	
	Mn.	Cl.	Mn.	Cl.
<i>Experiment 63</i>				
5.5	—	—	16.87	—0.94
7.5	14.7	—4.03	—	—
24.0	16.19	—0.9	17.6	6.0
48.0	17.15	5.99	21.86	12.23
72.0	26.53	27.6	25.6	30.4
96.0	44.49	36.66	39.2	—
120.0	53.2	51.8	48.15	56.4
144.0	57.86	61.7	57.00	63.0
168.0	62.6	68.26	65.6	66.06
192.0	71.0	71.0	69.78	72.08
<i>Experiment 59</i>				
5	22.13	—2.17	21.32	0.0
24	23.35	6.52	27.78	10.86
48	27.19	19.04	34.63	25.0
72	37.50	32.80	45.89	39.52
96	50.78	47.21	63.98	57.11
120	67.70	63.61	76.50	72.03
144	81.64	78.84	88.00	85.58
168	91.00	92.18	96.62	93.50
192	100.00	100.00	100.00	100.00

When oxygen is passed over the tissue for 24 hours its effects are similar to those observable when air is used and are shown by the data of Table IX.

TABLE IX

The Course of Absorption of Ions from 0.001 M. Manganese Chloride by Red Beet which has previously been treated with Oxygen (Expt. 58)

Time in hours.	Percentage absorption.			
	Untreated tissue.		Oxygen-treated tissue.	
	Mn	Cl.	Mn.	Cl.
5	24.13	—3.52	26.38	4.38
24	25.53	—1.49	31.74	7.82
48	30.03	7.13	37.83	27.19
72	38.29	28.72	55.42	50.22
96	57.66	48.80	73.00	70.24
120	68.30	—	91.84	88.82
144	78.76	76.82		
168	91.08	89.71		
192	97.00			

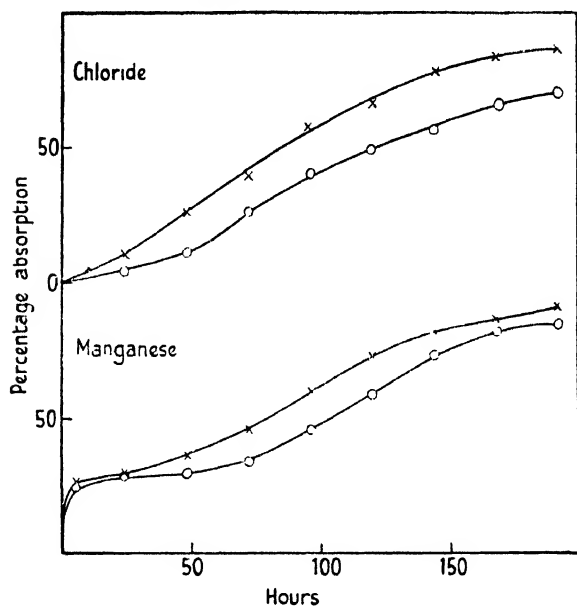


FIG. 7. The course of absorption by red beetroot tissue which has been previously treated with air of the ions of manganese chloride from a solution of an initial concentration of 0.001 M. \odot , untreated tissue; \times , air-treated.

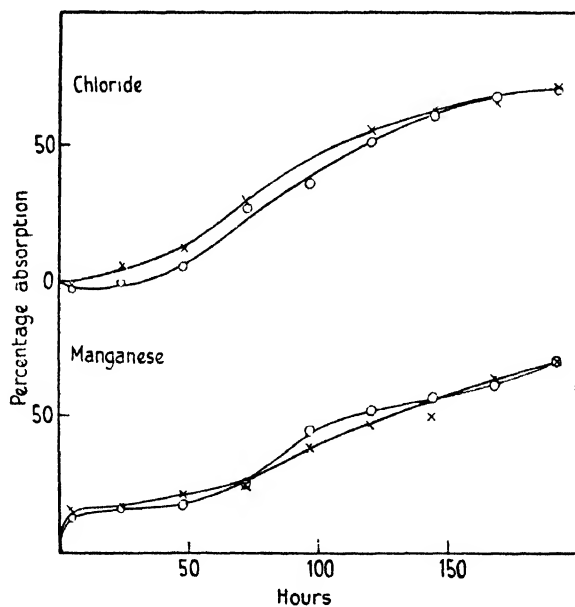


FIG. 8. The course of absorption by red beetroot tissue which has been previously treated with nitrogen of the ions of manganese chloride from a solution of an initial concentration of 0.001 M. \odot , untreated tissue; \times , nitrogen-treated.

TABLE X

Induced Absorption of Ions from 0.001 M. Manganese Chloride by Red Beet (as Percentage Quantity in the Original External Solution)

Hours immersed in MnCl ₂ .	Distilled water and gas pre-treatment.				Gas pre-treatment.			
	Manganese.		Chloride.		Manganese.		Chloride.	
	Air.	Nitrogen.	Air.	Nitrogen.	Air.	Nitrogen.	Air.	Nitrogen.
24	3.43	6.41	8.8	—1.76	1.9	2.87	6.05	5.58
48	11.6	6.59	25.54	1.96	7.77	6.08	15.39	6.09
72	35.1	23.82	40.11	14.67	11.76	8.73	22.80	4.76
96	28.8	17.71	29.61	19.21	14.42	4.85	17.76	9.12
120	21.09	5.67	22.83	12.71	14.25	2.17	18.10	5.96
144	10.49	4.79	22.28	8.32	8.64	3.25	7.88	4.02
168	12.99	5.86	35.8	3.78	4.82	4.3	8.39	0.06
Average daily increase.	17.64	10.12	26.44	8.41	9.08	4.41	13.77	5.0

The increased uptake of ions attributable to gas pre-treatment, the data for which were obtained from a number of experiments, are summarized in Table X, which shows the daily increased uptake as compared with the control in relation to the different methods employed. From these values the average daily increased absorption has been calculated, and will be seen when the gases are employed to be roughly twice when the discs are pre-treated in distilled water than when the gases are brought directly into contact with the tissue. It appears therefore that there are two factors operating when gases are utilized in distilled water, the effect of the respective gas and some other factor. Since immersion in distilled water for 24 hours removes the maximum amount of diffusible solutes, it would appear that this leaching effect of distilled water is this other factor which is conducive to enhanced intake of salt by the tissue.

V. The Effect of subjecting Red Beet Tissue to Different Temperatures on its Subsequent Uptake of Manganese Chloride

Observations on the possible effect of the temperature of tap-water on storage tissue with respect to subsequent salt intake are somewhat scanty. Steward and Berry (1943) suggested that the temperature of running tap-water may be one factor which exerts its influence on storage tissue. They further state that potato tissue loses potassium and no doubt other solutes to aerated solutions at the temperature of running tap-water (below 10° C.).

During the preparation of experiments of the present investigation, where tissue has been washed in tap-water, the average daily temperature of tap-water used has been recorded and may vary as follows. In expt. 69, conducted in May, the temperature was 14.5° C., while temperatures as low as 6.4° C. were recorded in January for experiments with artichoke; also the diurnal and nocturnal temperatures often varied by as much as 3° or 4° C.

Several recent researches on the respiration of storage tissue show quite

clearly that the respiratory activity of discs of storage tissue washed or kept at lower or higher temperatures varies considerably (Bennet-Clark and Bexon, 1943; Turner et al., 1947; Stiles and Dent, 1946, and others). Experiments were performed to ascertain if the uptake of solutes by storage tissue may be related to previous temperature effects.

Red beet tissue was used in all experiments. The conditions under which the temperature effect was applied varied slightly in two sets of experiments. In those performed in March the discs were placed on racks in tubes in constant temperature baths at various temperatures. Another method used was to keep the tissue in aerated distilled water at different constant temperatures.

Discs used in expts. 64 and 65 on March 3 and 10 respectively were well mixed and kept well moistened and aerated in tubes at temperatures of 10°, 20°, and 30° C. in replicate, for 24 hours, after which they were immersed in 0.001 M. manganese chloride and the course of absorption followed polarographically. Table XI and Fig. 9 summarize the results obtained.

TABLE XI

The Course of Absorption of Ions from 0.001 M. Manganese Chloride by Red Beet Tissue which has been previously subjected to Different Temperatures in Air for 24 Hours

Time in hours.	Percentage absorption.					
	Mn.			Cl.		
	10° C.	20° C.	30° C.	10° C.	20° C.	30° C.
<i>Experiment 64</i>						
5	11.68	15.8	19.9	4.28	2.86	—
24	15.10	18.44	28.6	8.56	20.0	—
48	27.50	31.62	41.74	30.0	37.19	—
72	42.90	44.75	63.85	48.6	45.82	—
96	58.00	62.64	72.16	63.30	58.28	—
120	74.20	75.58	80.72	77.18	68.06	—
<i>Experiment 65</i>						
5	18.64	19.40	17.20	—2.9	0.0	2.7
24	20.2	28.6	24.6	10.3	9.3	23.6
48	33.7	31.2	40.2	23.5	24.9	33.3
72	38.3	39.6	—	35.3	40.1	41.2
96	41.84	44.6	46.3	45.6	55.4	54.2
144	64.1	63.7	66.7	63.6	63.1	68.4
168	70.7	71.7	69.6	70.6	72.5	73.4

The results obtained illustrate that the effect of different temperature treatments do materially affect subsequent salt intake. The absorption of both ions appears to be accelerated where higher temperatures are employed. Nevertheless, this greater intake is not exhibited throughout the whole experiment; indeed, the course of absorption of anion, as illustrated in Table XI, expt. 64, and of the tissue treated at 10° C., gradually approaches and overtakes that of material given 20° C. of temperature, and after 96 hours

is in excess. A glance at Fig. 9 and Table XI shows that the different curves for cation absorption in expt. 64 gradually approach equality after 120 hours. These results suggested to the writer that action of higher temperatures on tissue might initiate some process which could cause a transitory increase in subsequent salt intake, depending on the time from the beginning of application of the temperature stimulus to the immersion of the tissue in salt solutions. In the subsequent experiments the time of the temperature application was doubled.

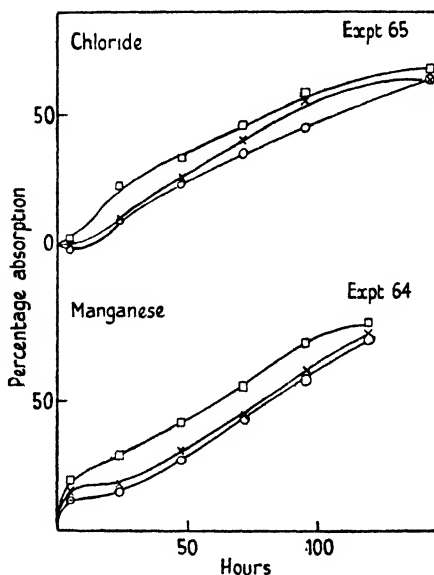


FIG. 9. The course of absorption by red beetroot tissue of the ions of manganese chloride from a solution of an initial concentration of 0.001 M. After preliminary treatment in air at 25° C. for 24 hours, at ○, 10° C.; ×, 20° C.; and □, 30° C.

Discs used in expt. 66 were cut on March 17 and treated similarly to the tissue used in expts. 64 and 65, except that the temperature treatment was extended to 2 days. Temperatures of 10°, 20°, and 30° C. were used.

In a later series of experiments, carried out in December, the temperatures used were lower—5°, 10°, and 15° C. for 48 hours—while the tissue was kept in aerated distilled water frequently changed during the treatment. Fig. 10 and Table XII summarize results obtained and indicate that length of temperature treatment does significantly affect the results. Where the temperatures employed were 10°, 20°, and 30° C., the initial enhanced uptake related to higher temperature is short, and is really only significant in the case of anion during the first 3 days, after which intake of tissue kept at 10° C. shows the maximum, that at 20° intermediate, while the discs treated at the highest temperature show the lowest relative intake. Results of an allied character are observable when the lower temperatures of 5°, 10°, and 15° C. were employed.

TABLE XII

The Course of Absorption of Ions from 0.001 M. Manganese Chloride by Red Beet Tissue which has been subjected to Different Temperatures for 48 Hours

Experiment 66: Treatment in air

Time in hours.	Percentage absorption.					
	Mn.			Cl.		
	10° C.	20° C.	30° C.	10° C.	20° C.	30° C.
5	19.2	22.0	21.6	2.0	3.7	10.0
24	25.8	25.8	25.6	8.2	12.2	18.5
48	31.4	33.2	35.2	22.8	29.2	28.8
72	45.2	—	40.8	31.4	39.0	38.6
96	53.0	48.7	50.0	51.6	45.7	42.8
120	66.0	59.2	56.4	62.6	58.3	55.1
144.3	76.5	73.2	64.8	76.0	71.1	65.3
168	84.4	80.3	75.0	87.0	77.1	73.7

Experiment 76: Treatment in aerated distilled water

Time in hours.	Percentage absorption.					
	Mn.			Cl.		
	5° C.	10° C.	15° C.	5° C.	10° C.	15° C.
5.0	19.4	19.4	20.0	0.0	9.8	3.8
24.2	22.5	22.0	22.3	3.9	17.0	9.6
48.0	29.2	29.3	31.8	21.8	31.7	29.0
71.0	37.6	42.7	36.4	37.8	46.4	35.5
120.0	—	—	41.2	—	—	—
145.3	56.2	60.0	44.7	62.4	73.4	56.1
170.0	72.0	73.2	50.5	74.1	88.3	63.7

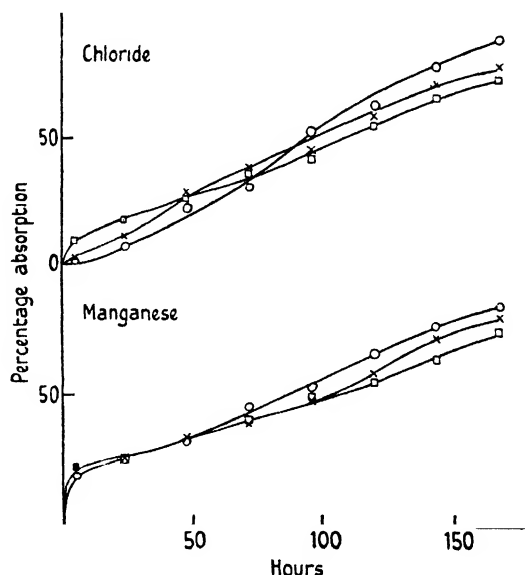


FIG. 10. The course of absorption by red beetroot tissue of the ions of manganese chloride from a solution of an initial concentration of 0.001 M. After preliminary treatment in air at 25° C. for 48 hours, at ○, 10° C.; ×, 20° C.; and □, 30° C.

DISCUSSION OF EXPERIMENTAL RESULTS

The experiments described in the preceding section clearly illustrate that the absorption of manganese by storage tissue is materially affected by the different pre-treatments employed. The effect of running tap-water, distilled water, air, oxygen, nitrogen, and temperature were selected because it was considered that these might be regarded as uncontrolled variables which might be operating to a greater or less extent during some methods of tissue preparation which have been used in the past. While the inevitability of such processes as tissue washing and handling is realized as an essential part of the preparation of tissue for salt uptake experiments, nevertheless it is emphasized that as carefully controlled conditions as possible should be maintained when these operations are in process. Particularly is this necessary when tap-water treatment is used, where temperature fluctuations are so varied, as has already been noted.

Experiments with parsnip have shown that this tissue is particularly sensitive to washing in running tap-water, agreeing in this respect with beet, carrot, and potato. Although no satisfactory explanation is yet forthcoming to account for this phenomenon, Stiles and Dent (1946) have pointed out that the enhanced intake of tissue so washed may be related to the increased metabolic activity of the tissue exposed for a long period to air during washing. Their researches on respiration clearly demonstrate a gradual rise in respiratory activity of storage tissue over a period of some 200 or more hours during the washing process.

From the data given in this paper, when air is excluded from running tap-water during the washing of parsnip tissue it is evident that continuous contact of the tissue with the oxygen in tap-water does significantly increase its subsequent salt uptake. But the intake is also favoured by prolonged washing in the absence of oxygen, suggesting that there is some further factor associated with running tap-water treatment affecting and enhancing salt uptake. The possibility of the removal of solutes from the tissue during washing has been suggested here, and although this explanation was not favoured by Asprey (1937), Steward and Berry (1943) point out that its effect cannot be neglected.

Attention has already been drawn to the fact that prolonged immersion of storage tissue in distilled water at 25° C. is similar in effect to immersion in running tap-water, while it has also been shown that treatment with air and nitrogen of tissue kept in distilled water for 24 hours is more favourable to salt uptake than the effect after these gases have been applied to the moist tissue under similar temperature conditions. The effect of the employment of air and distilled water on further subsequent absorption is nearly double that resulting when the gas is employed alone. A similar relationship is found when access of the tissue to air is excluded, the values for induced absorption of manganese by beet tissue being 10·12 when nitrogen and distilled water are employed, as compared with 4·41 when the gas is used alone. It seems

that this extra-absorption may be attributed to a previous loss of electrolytes in distilled water by the tissue. That one effect of running tap-water is to cause a continuous loss of solutes can only be suggested at this stage. Detailed analyses of the mineral content of tissue subjected to continuous washing are necessary. A more detailed analysis of the contents of artichoke discs before and after washing might do much to explain why this tissue behaves differently from others investigated, as no satisfactory explanation can be given at present.

The data obtained, using different temperature effects on beet, are instructive, since they serve to show how sensitive storage tissue may be to previous temperature treatment, while emphasizing the need for controlled and uniform temperature conditions during preparation, especially when washing. Considering the individual experiments, it is clear that increased temperature treatment does not necessarily mean enhanced absorption. The resulting course of absorption appears to depend on at least two factors, the time during which the respective temperature is employed and the degree of temperature used. It is considered that this temperature effect may in some way be linked with respiration, and attention is now drawn to some recent results obtained by Stiles and Dent (1946) which may shed some light on this question.

These authors show that the peak of respiratory activity of red beet tissue kept in tap-water at about 12° C. is not reached until about 200 hours after cutting, whereas discs kept at 25° C. reach their respiratory peak after some 18 hours. It appears that the time required to attain maximum respiration is related to temperature; also, that at any given time, after 24 hours from time of cutting, tissues kept at different temperatures may be expected to exhibit different respiratory intensities. Those subjected to higher temperatures will have passed their peak of activity and may be falling towards, or have attained, an approximately level plateau of respiration. On the other hand, the respiratory intensity of tissues kept at lower temperatures may not have yet reached their peaks but be exhibiting an upward movement, while a time will be reached when the rising rate will approach that falling and indeed overtake it. The time taken for coincidence of respiratory intensities of different temperatures will depend on the actual temperatures employed.

As has already been pointed out, there is a similar relationship between the effect of temperature treatment and subsequent absorption, which appears to be connected with a time factor. This suggestion, that temperature may exert its effect on subsequent salt intake by virtue of its effect on the respiratory intensity, can be only speculative at this stage. Respiratory data coupled with salt-absorption figures are necessary to elucidate this point.

Assuming, however, that the respiratory intensity of the tissue immediately before immersion in the salt solution is a factor in determining the rate and course of salt absorption, it would appear reasonable to allow salt absorption to begin when the tissue has reached a steady level of respiratory activity. In addition the conditions of washing should be at a controlled temperature of such magnitude that the peak of respiration is eliminated as soon after cutting

as possible. It appears from unpublished data kindly supplied by Mr. A. D. Skelding that with red beet kept at 20° C. this period is reached after about 33 hours from cutting.

SUMMARY

1. The effect of various methods of treating storage tissue on its subsequent absorption of 0.001 M. manganese chloride has been examined and certain conclusions regarding the individual treatment effects drawn.

2. The effect of prolonged previous washing of parsnip tissue on salt intake is similar to that previously recorded for other tissues such as carrot and beetroot where an enhanced uptake of both ions is facilitated. Artichoke tissue differs in this respect from other tissues examined, in that its salt absorption is not favoured by prolonged washing.

3. The increased uptake of both ions of manganese chloride by parsnip tissue is favoured by prolonged washing in tap-water from which air is excluded.

4. Pre-immersion of red beet tissue in distilled water is similar in its effect to pre-immersion in tap-water, the effect of enhanced intake being increased with longer immersion.

5. Red beet tissue treated with air and oxygen absorbs manganese and chloride more rapidly than the untreated control. Nitrogen is similar in its effect, although to a less extent. The effect of air and nitrogen in favouring absorption is greater when applied to the tissue in distilled water than when the gases are brought into contact with the moist tissue. It is suggested that the leaching effect of distilled water may account for this disparity.

6. It is considered that aerated tap-water may induce higher salt uptake because of two effects, that of its oxygen content and by continuous removal of electrolytes from the washed tissue.

7. The effect of subjecting red beet tissue to different temperatures on its subsequent intake of manganese chloride depends on at least two factors—the time of temperature application and the degree of temperature employed. An increase in the time of temperature application favours the intake by tissues receiving a lower temperature stimulus. Stress has been laid on the course of respiratory intensity by red beet at different temperatures, with particular reference to the time taken to reach peak and plateau respiration levels at different temperatures, while it seems likely that further correlation with these respiration data may satisfactorily explain salt uptake as conditioned by previous temperature effects.

8. In the light of results obtained, especially those with temperature, the employment of controlled conditions of tissue preparation is emphasized, particularly during washing, which it is suggested be carried out at a temperature which will eliminate the peak of respiratory intensity as soon as possible after cutting without damaging the tissue and for which purposes temperatures in the neighbourhood of 20° C. are proposed.

In conclusion, thanks are due to Professor W. Stiles for his suggestions and guidance. Also to the Research Committee of the University of Birmingham and the Government Grants Committee of the Royal Society, whose grants made the purchase of apparatus used in this work possible.

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Jaccard's Generic Coefficient and Coefficient of Floral Community, in relation to the Logarithmic Series and the Index of Diversity

BY

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IN a series of papers published between 1902 and 1941 Paul Jaccard, largely as a result of the study of the flora of Alpine Valleys in Switzerland, developed two concepts in connexion with the structure of plant communities, which he called 'the Generic Coefficient' and the 'Coefficient of Floral Community'.

The 'generic coefficient' was defined as the number of genera required to yield one hundred species at the same average number of species per genus as in the sample available.

Thus, if there are S species and G genera in the particular portion of the association or community under study,

$$\text{the generic coefficient} = 100 \frac{G}{S}.$$

In other words, it is 100 times the reciprocal of the average number of species per genus (which latter value he calls the 'generic quotient'). He uses it as a measure of the generic diversification of the species contained in the association. For example, if two areas each contained 50 species, the first representing 30 genera, and the second 40 genera, his coefficients would be 60 and 80; the higher the coefficient the greater the generic diversity.

Jaccard's 'coefficient of floral community', on the other hand, was intended as a measure of relationship between two different samples, such as two quadrats of similar area; in fact he defines his quadrats to be of one square metre. He defines it as

$$100 \times \frac{\text{The number of species common to the two quadrats.}}{\text{The total number of species on the two quadrats.}}$$

For example, if two quadrats, each of one square metre, had 31 species in all, of which 23 were common to both, the coefficient would be $100 \times 23/31 = 74.2$ per cent. The higher the value of the coefficient the closer the similarity between the two samples.

If there are several quadrats Jaccard calculated all the possible combinations in pairs and gave an average coefficient for the series.

Jaccard's 'coefficient of floral community' is dependent on the relation between numbers of individuals and numbers of species in the communities

to be compared. His 'generic coefficient', on the other hand, is dependent on the number of species and the number of genera in one particular community; but it can also be used to compare conditions in different communities.

Maillefer in 1929 criticized Jaccard's generic coefficient and said that it depended on the size of the sample or the area under consideration.

Jaccard in 1941 replied to some of Maillefer's criticisms and suggested that the size of the sample alone was insufficient to account for the differences and resemblances found in the generic coefficients of different plant communities, and restated his claim that the coefficient indicated real ecological properties of the floras.

In recent years it has been found that in both animal and plant population the frequency distribution of genera with different numbers of species is in a mathematical form which can be closely represented by the logarithmic series (see Williams, 1944 and 1947*a*). It has been also found that in animal populations, and probably in plant populations, the number of species with different numbers of individuals is represented by the logarithmic series (see Fisher, Corbett, and Williams, 1943; Williams, 1944 and 1947*b*).

The logarithmic series can be written in two ways:

either
$$n_1; \frac{n_1}{2}x; \frac{n_1}{3}x^2; \frac{n_1}{4}x^3; \dots,$$

or
$$\alpha x; \alpha \frac{x^2}{2}; \alpha \frac{x^3}{3}; \alpha \frac{x^4}{4}; \dots$$

In each case the first term, n or αx , is the number of groups with one unit (i.e. species with one individual, or genera with one species), the second term is the number of groups with two units, and so on. In the series x is a constant (for the sample) less than unity; and α is a constant for all samples of whatever size from the same association, and we have called it the 'Index of Diversity'.

The logarithmic series is convergent and gives a finite sum for both groups and units. If we know the total number of groups and of units in any one sample we can calculate not only n , x , and α , and hence the whole series, but also the number of groups for any larger or smaller sample selected at random from the same population.

For example, in a random sample of an insect population, made by means of a trap, there were 15,600 insects representing 240 species. It follows from this (for methods of calculation see Williams, 1947*b*) that n_1 is approximately 40 (i.e. 40 species are each represented by a single individual), $x = 0.99743$, and α is also approximately 40.

The conception of this 'Index of Diversity' has proved to be of very considerable ecological interest. It is a property of the population sampled, and not of the sample, and it is a measure of the extent to which the units are associated into groups, or the groups divided into units. It is high when there

is great diversity, e.g. a large number of species for the numbers of individuals. Considerable discussion on it will be found in Fisher, Corbett, and Williams (1943) and in Williams (1944 and 1947a).

One of the properties of a population in which the units and groups are arranged in a logarithmic series is that, except for very small samples, the number of groups represented in a sample is proportional to the logarithm of a number of units. If, for example, a sample from a particular population consists of 1,000 individuals with 100 species, then each time the number of individuals is doubled, approximately 19 species will be added. Thus 2,000 individuals will give 119 species, 4,000 individuals 138 species, 8,000 individuals 157 species, and so on.

When an attempt is made to transfer these theories of population structure on the individual-species level from animals to plants, we find two important differences, one making things easier and the other more difficult. In animal populations, and particularly in flying animals such as insects, it is often difficult to know whether a particular individual found in a sample really belongs to the community being studied or is merely a casual visitor, here to-day and gone to-morrow. In plants this difficulty does not occur; each plant has a definite location from which it does not move.

On the other hand, it is with animals very easy to say that a sample contains a particular and definite number of individuals—each individual is a clear-cut entity. But in plants this is not so; it is very difficult in many cases to say where an 'individual' begins and ends, particularly when you get vegetative reproduction by stolons, tillers, &c.

It is, however, possible to a certain extent to evade this difficulty by making the assumption that, in a series of random samples of different sizes from a single plant association, the number of 'individuals', or perhaps better 'plant units', is proportional to the area of the sample. We can then avoid this problem of the actual number of units by comparing two or more samples.

Where a study is made of the number of species of plants represented in areas of different sizes in the same association (Williams, 1944) it is found that, within close limits, the number of species present is proportional to the logarithm of the area of the sample. It will be seen, therefore, that there is evidence of the existence in plant populations of exactly the same type of individual-species structure as has been found in animals—a structure which can be closely represented by the logarithmic series.

This leaves us in a position to discuss the two coefficients suggested by Jaccard on the assumption that the logarithmic series can be used to represent the structure of the individual-species and the species-genera relations in plant communities, and also that the conception of the 'Index of Diversity' as a measure of richness is applicable to plant populations.

Jaccard's 'Generic Coefficient'

The relation between the frequency of genera with different number of species has already been shown to be closely represented by a logarithmic

series both in animals and plants. For plants evidence is brought forward in Williams, 1944, pp. 23-32, for the flowering plants of the world, and for British flowering plants in both Bentham and Hooker's and Babington's classifications; also in Williams (1947a) for the genera and species in comparatively small plant communities.

On the assumption that this interpretation is sound it can be shown that the average number of species (S) per genus (G) in a sample from a population based on a logarithmic series with Index of Generic Diversity α is given by¹

$$S/G = \frac{e^{G/\alpha} - 1}{G/\alpha}.$$

Therefore Jaccard's coefficient = $100 \times \frac{G/\alpha}{e^{G/\alpha} - 1}$.

In other words, his coefficient is dependent on the ratio between the number of genera represented in the sample and the richness of the population sampled. If α remains constant, i.e. if a series of samples of different sizes are taken from the same community, the coefficient varies with the size of G , that is, with the size of the sample.

If, on the other hand, samples with the same number of genera are taken from two communities with different value of α , then the coefficient will vary with α .

For example, if a sample containing sufficient species to represent 20 genera is extracted from a population with an Index of Generic Diversity of 10, the average number of species per genus will be

$$\frac{e^2 - 1}{2} = \frac{6.4}{2} = 3.2$$

and Jaccard's coefficient would be 31.

If a sample sufficiently large to contain 40 genera was selected from the same association, the average number of species per genus would be

$$\frac{e^4 - 1}{4} = 13.5$$

and Jaccard's coefficient would be 7.7.

Jaccard's coefficient is therefore a double function depending on the generic richness of the population and also on the size of the sample taken. It is therefore not so good a measure of the ecological structure of the population as the Index of Diversity alone.

Jaccard's 'Coefficient of Floral Community'

It can be shown that the number of species (S) represented by a number of individuals (N) taken by a random sample from a population arranged in a logarithmic series with the Index of Specific Diversity α is given by

$$S = \alpha \log_e \left(1 + \frac{N}{\alpha} \right).$$

¹ e is the base of the Napierian logarithms = 2.71828.

The number of species in a sample twice the size, or in two samples of the same size, is therefore

$$\alpha \log_e \left(1 + \frac{2N}{\alpha} \right).$$

If N is large compared with α , as it should be for a good representative sample, it is possible to neglect the 1 in comparison with N/α , and from this it can be shown (see Williams, 1947b, p. 269) that the increase in number of species by doubling a sample is equal to $\alpha \log_e 2 = 0.69\alpha$.

Thus, if two quadrats of the same size (say one square metre as defined by Jaccard) are taken from a population, which is arranged in a logarithmic series, and if each contains S species, then the two together will contain $S + \alpha \log_e 2$ species. It follows that each quadrat must contain on an average $\alpha \log_e 2$ species not found in the other quadrat, and therefore the number common to the two quadrats must be

$$S - \alpha \log_e 2.$$

It is interesting to note that this is actually the number of species found on half a quadrat.

Thus, Jaccard's Coefficient of Floral Community

$$= 100 \frac{S - \alpha \log_e 2}{S + \alpha \log_e 2} \quad \text{or} \quad 100 \times \frac{S - 0.69\alpha}{S + 0.69\alpha}.$$

It is thus dependent upon S , the number of species in one quadrat, which is in turn dependent on the size of the quadrat, or the number of plant units it contains; and also on α , the Index of Specific Diversity of the population.

If within the same population the sample size is increased, Jaccard's coefficient rises; if the same-sized samples are taken from a richer flora, the coefficient falls.

The following is a numerical example:

In a population with an Index of Specific Diversity of 10 if 1 square metre contains 46 species

then 2 square metres will contain 52 species approximately

10	“	“	“	69	“	“
20	“	“	“	76	“	“

Thus for two areas of 1 square metre Jaccard's coefficient

$$= \frac{(2 \times 46) - 52}{52} \times 100 = 77 \text{ per cent.},$$

but for two areas of 10 square metres the coefficient would be

$$\frac{(2 \times 69) - 76}{76} 100 = 82 \text{ per cent.}$$

Even with Jaccard's restriction of quadrat area to 1 square metre this difficulty is not eliminated, as it is the number of plant units in the quadrat which must be kept constant, and the number of plant units in quadrats of the same area is not the same in different associations.

Jaccard's coefficient can also be written as

$$100 \times \frac{\log_e(1 + \frac{1}{2}(N/\alpha))}{\log_e(1 + 2(N/\alpha))}$$

when N is the number of 'plant units' per quadrat.

It is thus apparent that all pairs of quadrats with the same N/α ratio will give the same coefficient.

In any community which has an individual-species structure in the form of a logarithmic series Jaccard's 'Coefficient of Floral Community' is thus also a measure of two factors: one, N is a property of the size of the sample; the other, α , or the Index of Diversity, is a property of the population sampled. The coefficient contains no information that is not more clearly stated by the Index of Specific Diversity alone.

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Studies on the Biological Activity of Griseofulvin

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With two Figures in the text

I. INTRODUCTION

A METABOLIC product of the mould *Penicillium janczewskii*, capable of causing profound disturbances in the hyphal development of *Botrytis allii* and other fungi, was isolated in pure form (Brian, Curtis, and Hemming, 1946) and named 'curling factor'. This substance was subsequently shown (Grove and McGowan, 1947; Brian, Curtis, and Hemming, 1948) to be chemically and biologically identical with a metabolic product of *Penicillium griseofulvum* isolated and described by Oxford, Raistrick, and Simonart (1939) under the name of griseofulvin. Griseofulvin is unusual among biologically produced substances in that it contains chlorine ($C_{17}H_{17}O_6Cl$); it is now known to be produced by three species of *Penicillium*—*P. patulum*, *P. griseofulvum*, and *P. janczewskii*.

The effect of griseofulvin on the development of *Botrytis allii* hyphae is characteristic. In concentrations of the order of 10 $\mu g./ml.$ it causes the production of highly stunted and gnarled germ-tubes, usually with spatulate extremities, whose development ceases at an early stage; lower concentrations (1 $\mu g./ml.$) cause excessive branching and distortion of the hyphae, and still lower concentrations (0.1 $\mu g./ml.$) cause a marked spiral wave of the hyphae without noticeable reduction in the growth rate. A further notable feature is that relatively high concentrations (50–100 $\mu g./ml.$) do not prevent germination of *Botrytis allii* conidia, though far lower concentrations cause the morphogenetic disturbances described. Preliminary observations (Brian, Curtis, and Hemming, 1946) indicated that the effects of griseofulvin on other fungi were qualitatively similar. The present communication is intended to amplify this statement by describing the physiological effects of griseofulvin on a wider range of organisms in addition to presenting a more detailed analysis of its effects on *Botrytis allii*.

II. STABILITY OF AQUEOUS SOLUTIONS OF GRISEOFULVIN

An essential preliminary to further investigation of the activity of griseofulvin was some knowledge of the stability of aqueous solutions. Solutions (40 $\mu g./ml.$) were made up in McIlvaine's citric acid-phosphate buffer (the requisite amount of griseofulvin was dissolved in 2 ml. ethanol and made up

to 100 ml. with buffer) at pH levels of 3.0, 5.2, 7.1, and 8.8, stored at 25° C., and assayed at intervals. The method of assay has been previously described (Brian, Curtis, and Hemming, 1946); briefly, it involves making serial dilutions in a nutrient and observing germination of *Botrytis allii* conidia in these dilutions. The greatest dilution causing marked stunting and the greatest dilution causing distortion or spiral waving are recorded. Results of this experiment are given in Table I; a recording coded as $S_{16}C_{256}$ indicates that marked stunting was observed in dilutions down to 1:16 and waving down to 1:256. Allowing for the subjective error in this assay, it is obvious that griseofulvin is highly stable within this pH range, which covers all experimental requirements. Samples of these solutions were also autoclaved for 20 min. at 20 lb./sq. in. without appreciable loss of activity. It is reasonable to conclude that griseofulvin solutions in culture media can be autoclaved without fear of loss of activity and that in experiments of long duration the concentration of griseofulvin will remain relatively constant.

TABLE I

Bio-assays of Griseofulvin Solutions in McIlwaine's Buffer

Period of storage (days).	pH 3.0.	pH 5.2.	pH 7.1.	pH 8.8.
0	$S_{16}C_{256}$	$S_{16}C_{512}$	$S_{16}C_{256}$	$S_{16}C_{256}$
1	$S_{16}C_{256}$	$S_{16}C_{512}$	S_8C_{256}	S_8C_{256}
2	S_8C_{128}	S_8C_{128}	S_8C_{128}	S_8C_{128}
6	S_8C_{128}	S_8C_{128}	S_8C_{128}	S_8C_{128}
9	S_8C_{128}	S_8C_{128}	$S_{16}C_{128}$	$S_{16}C_{128}$
15	$S_{16}C_{128}$	$S_{16}C_{256}$	$S_{16}C_{128}$	$S_{16}C_{256}$
22	S_8C_{128}	S_8C_{256}	$S_{16}C_{256}$	$S_{16}C_{256}$
29	S_8C_{256}	S_8C_{256}	S_8C_{128}	$S_{16}C_{128}$

III. PHYSIOLOGICAL EFFECTS OF GRISEOFULVIN ON FUNGI

(a) *Morphogenetic effects on representatives of main groups of fungi*

Griseofulvin was included in prune agar at levels of 0, 1, 5, 10, and 20 µg/ml. The fungi listed in Table II were grown on this medium in Petri dishes at 25° C. Observations were made on gross cultural appearance, the rate of radial growth of colonies was measured, and the growing edge of the colonies was examined under a low-power microscope. The effect of griseofulvin was, in general, to reduce the rate of radial growth and to cause morphogenetic changes of the type already described for *Botrytis allii*, the most noticeable features being excessive branching, distortion, and spiral waving and thickening of the hyphae. Not all fungi were equally sensitive; in Table II are recorded the least concentrations causing an obvious reduction in the growth rate and the least concentrations causing an obvious morphogenetic response.

By far the most striking feature of these results is the sharp distinction in response between the Oomycetes and all other classes of fungi examined. The Oomycetes showed no morphogenetic response to griseofulvin whereas all the remaining fungi showed a typical response. A number of exceptions

to this generalization may be noted here; the two yeasts studied (*Saccharomyces cerevisiae* and *Torulopsis utilis*) showed no response and the Oomycete fungus *Achlya radiosa* showed a slight reduction in growth rate in the presence of 20 µg./ml. griseofulvin but without any morphological response. These broad general differences in response to griseofulvin are discussed in a later section (VI) of this paper.

TABLE II

Least Concentrations of Griseofulvin (µg./ml.) required to produce a 50% Reduction in Growth Rate and a Microscopically Visible Morphological Response

Fungus.	Cellulose test.	Growth reduction.	Morphological response.
PHYCOMYCETES			
OOMYCETES			
<i>Achlya radiosa</i> Maurizio	+	20 ¹	> 20
<i>Phytophthora cactorum</i> (Leb. & Cohn) Schrecht.	+	> 20	> 20
„ <i>cryptogea</i> Pethybridge	+	> 20	> 20
„ <i>erythroseptica</i> Pethybridge	+	> 20	> 20
„ <i>palmivora</i> Butler	+	> 20	> 20
<i>Pythium aphanidermatum</i> (Eds.) Fitz.	+	> 20	> 20
„ <i>intermedium</i> de Bary	+	> 20	> 20
„ <i>mammilatum</i> Meurs	+	> 20	> 20
„ <i>ultimum</i> Trow.	+	> 20	> 20
<i>Saprolegnia</i> sp.	+	> 20	> 20
ZYGOMYCETES			
<i>Absidia glauca</i> Hagem (+ strain)	—	5	1
„ „ „ (— strain)	—	5	1
<i>Mucor erectus</i> Bain.	—	20	10
„ <i>mucedo</i> Bref. (+ strain)	—	5	5
„ „ „ (— strain)	—	5	5
<i>Syncephalastrum racemosum</i> (Cohn) Schrecht.	—	10	1
<i>Thamnidium elegans</i> Link.	—	1	5
ASCOMYCETES			
<i>Byssosclamyces fulva</i> Olliver & Smith	—	10	1
<i>Chaetomium globosum</i> Kunze	—	10	1
<i>Diaporthe pernicioso</i> (Ell. & Ev.) March.	—	1	1
<i>Gibberella saubinetii</i> (Mont.) Sacc.	—	1	1
<i>Neurospora sitophila</i> (Mont.) Sacc.	—	5	5
<i>Saccharomyces cerevisiae</i> Hansen	—	> 20	> 20
BASIDIOMYCETES			
<i>Armillaria mellea</i> (Vahl.) Quel.	—	1	1
<i>Coniophora cerebella</i> Alb. & Schw.	—	> 20	5
<i>Echinodontium tinctorum</i> Ell. & Ev.	—	20	5
<i>Fomes fomentarius</i> (L.) Fr.	—	5	5
<i>Hydnum coralloides</i> Scop.	—	20	10
<i>Lentinus lepidus</i> Fr.	—	10	10
<i>Polystictus versicolor</i> (L.) Fr.	—	10	5
<i>Stereum purpureum</i> Fr.	—	5	1

¹ Reduction in growth c. 30 per cent. only.

TABLE II (cont.)

Fungus.	Cellulose test.	Growth reduction.	Morphological response.
FUNGI IMPERFECTI			
<i>Aspergillus amstelodami</i> (Mang.) Thom & Church	—	10	5
„ <i>niger</i> v. Tiegh.	—	10	5
<i>Botrytis allii</i> Munn.	—	1	1
„ <i>cinerea</i> Pers.	—	1	1
„ <i>narcissicola</i> Klebahn	—	1	1
„ <i>tulipae</i> (Lib.) Lind.	—	1	1
<i>Endomycopsis albicans</i> (Vuill.) Dekker	—	5	5
<i>Fusarium caeruleum</i> (Lib.) Sacc.	—	5	1
<i>Helminthosporium avenae</i> Eidam	—	20	1
<i>Myrothecium verrucaria</i> (Alb. & Schw.) Ditmar	—	5	1
<i>Penicillium expansum</i> Link.	—	20	10
„ <i>janczewskii</i> Zal.	—	10	10
„ <i>notatum</i> Westling	—	5	5
<i>Phoma betae</i> Frank	—	1	1
<i>Stachybotrys atra</i> Corda	—	10	1
<i>Torulopsis utilis</i> (Henn.) Lodder	—	> 20	> 20
<i>Trichoderma viride</i> Pers. ex Fr.	—	5	5
<i>Trichothecium roseum</i> Link.	—	10	5
<i>Verticillium alboatrum</i> Reinke & Berth.	—	10	1
„ <i>cinnabarinum</i> (Cda.) Reinke & Berth.	—	1	1

Smaller differences in the degree to which griseofulvin affected morphogenesis could be seen among the sensitive classes of fungi; these differences are of smaller significance and are probably associated with differences in permeability and general metabolic vigour. More detailed observations, not recorded in Table II but worthy of note, are given below:

Syncephalastrum racemosum normally increases in colony size by two distinct processes, (a) by radial growth of the hyphae within the medium, and (b) by production of aerial stolons, the distal ends of which fall on to the surface of the agar and form radially expanding mycelia (appressoria) in advance of the radial edge of the primary colony. Normally the mycelia of the primary colony and of the various appressoria eventually become interwoven and indistinguishable in a mature Petri-dish colony. In the presence of griseofulvin the growth rate of the submerged mycelia of the primary colony and of the appressoria is much reduced but the growth rate of aerial stolons is not affected. Consequently the original colony and the various appressoria do not fuse, resulting in a striking colony-form, consisting of numerous separate restricted appressoria linked by aerial stolons. This observation is of some significance as it indicates that griseofulvin is not translocated within the hyphae but only affects hyphae in intimate contact with a griseofulvin-containing medium.

Absidia glauca showed a similar, though less marked, response.

Diaporthe pernicioso was very sensitive to griseofulvin and hyphae were much distorted and growth generally disorganized by a concentration of 1 µg./ml.

Gibberella saubinetii reacted to the presence of griseofulvin, by the type of

morphological response already described, and also by the production of numerous intercalary swellings somewhat similar in appearance to chlamydo-spores, but lacking the thickened cell-wall.

Armillaria mellea, in prune agar containing no griseofulvin, produced rhizomorphs abundantly, 2–5 cm. in length. In the presence of 1–10 $\mu\text{g./ml.}$ griseofulvin these were much reduced in size, not exceeding 0.25 cm. in length, and in the presence of 20 $\mu\text{g./ml.}$ griseofulvin formation of rhizomorphs was completely inhibited.

Penicillium janczewskii is of particular interest, being one of the fungi known to produce griseofulvin in quantity. Under the conditions of this experiment, growing on prune-agar containing griseofulvin, this fungus showed a small but definite response. The normal colony of *P. janczewskii*, if growing on a suitable medium for griseofulvin production, is composed of highly distorted hyphae and is restricted in growth. This is seen particularly on Czapek-Dox agar. If grown on a medium unsuitable for griseofulvin production the hyphae are normal in appearance and the colonies are of a more freely spreading type; this is seen well on media containing ammonia-nitrogen and no chloride. On such a medium the response to added griseofulvin is more marked.

After 7 days' growth mycelial transfers were made from the edge of colonies of all the fungi, from each of the media, to a similar prune-agar medium containing no griseofulvin. There was a slight initial check in the case of some transfers from media containing the higher concentrations of griseofulvin, but after this growth was normal in every way. This initial check can probably be attributed to small quantities of griseofulvin taken over with the inoculum. The effect of griseofulvin is thus purely transitory, being exhibited only when hyphae are directly exposed to the substance.

(b) *Effect on growth rate of Botrytis allii*

Spore germination tests have been most commonly used in recent years for quantitative studies of toxic action on fungi, and the theoretical implications of such tests have received much attention. Such a technique could not be used in the study of griseofulvin since, within the range of concentrations allowed by the low water-solubility of griseofulvin, it does not prevent germination, though it has a marked effect on subsequent hyphal extension. The toxicity of griseofulvin was therefore studied by its effect on hyphal extension in agar media. For this purpose the growth-rate tubes devised by Ryan, Beadle, and Tatum (1943) were used. The theory of this method has received little attention. It must be borne in mind that the effect of griseofulvin on growth rate is complex, being a summation of several processes. These include a direct effect on the rate of hyphal extension and an indirect effect on growth rate, as measured by radial spread of a colony, due to the distorted and devious path of extension shown by hyphae in the presence of griseofulvin. Griseofulvin was included in Czapek-Dox and Raulin-Thom media in concentrations of 0, 0.2, 0.5, 1.0, and 5.0 $\mu\text{g./ml.}$; Figs. 1 and 2 show the rate of growth of *Botrytis allii* on these media.

The curves relating size of colony to age (Figs. 1A and 2A) indicate a broad similarity of behaviour on the two media. A short initial lag is followed by a phase of linear growth rate, but whereas on Raulin-Thom this growth rate is maintained until the end of the experiment, on Czapek-Dox there is a gradual slight falling-off in rate, i.e. this medium is a 'staling' medium (Brown, 1923) for *Botrytis allii*. The relation between growth rate and dose of griseo-

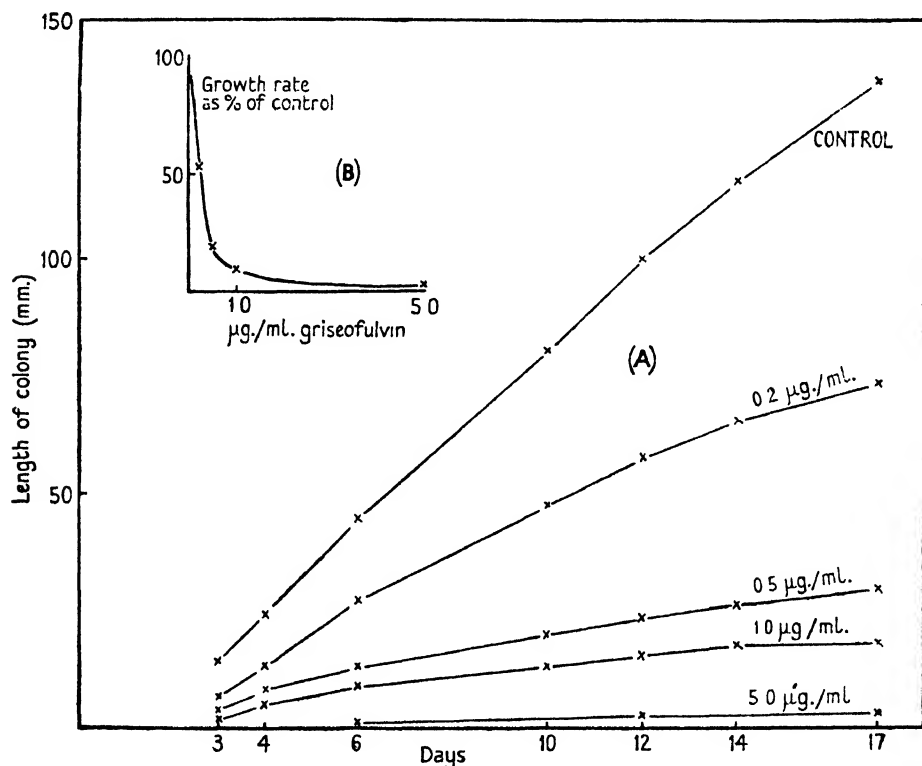


FIG. 1. The effect of griseofulvin on the growth in length of *Botrytis allii* on Czapek-Dox medium.

fulvin is shown in Figs. 1B and 2B; mean growth rate was calculated on the basis of periods in which growth rate was relatively constant, i.e. during the period 6–12 days for colonies on Czapek-Dox, 6–17 days for colonies on Raulin-Thom. The same general type of dose/response relationship is shown on both media. The dose/response relationships are very similar to those described for much simpler toxic agents, such as phenol or *p*-hydroxybenzoate, by Vincent (1947), in that the relations response/log dose and log response/dose were not linear. Without laying too much stress on the significance of the relation, it may be noted that the relation log dose/log response is linear, as was also found by Vincent for simple toxic agents; thus griseofulvin shows no unusual properties in its dose/response relationships.

These data emphasize the high activity of griseofulvin; in the presence of

only 0.2 $\mu\text{g./ml.}$ growth rate is halved, although at this concentration morphogenetic disturbances are not severe. In a griseofulvin concentration of 5 $\mu\text{g./ml.}$ growth virtually ceases.

(c) Effect on oxygen uptake by *Botrytis allii*

It was found convenient to measure oxygen uptake with a Barcroft differential manometer (direct method). Mycelium for the experiment was produced by growing *Botrytis allii* for 5 days on liquid Czapek-Dox medium (in

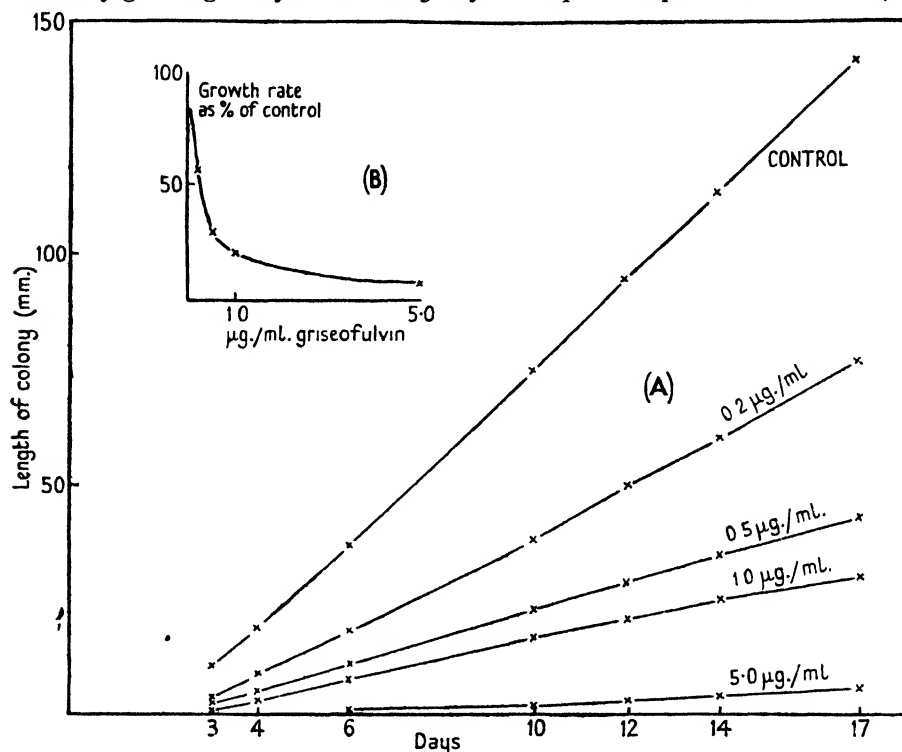


FIG. 2. The effect of griseofulvin on the growth in length of *Botrytis allii* on Raulin-Thom medium.

later experiments 0.05 per cent. yeast extract was added to the medium, producing stronger growth and mycelium with a higher Q_{O_2}). The mycelial felt, which was thin and of a loose texture, was then removed and washed in two changes of sterile distilled water. One or two 1-cm. discs cut from the felt were added to the Barcroft flasks or, alternatively, a mycelial suspension produced in a blender was used; in either case the dry weight of mycelium used was of the order of 15–30 mg. Preliminary experiments indicate that the rate of oxygen uptake was independent of the rate of agitation, i.e. there were no diffusion effects.

Table III shows the oxygen uptake by *Botrytis allii* in Weindling medium (1.25 per cent. glucose), pH 3.5, at 25° C. In these experiments discs from mycelial felts were used and griseofulvin or azide was added to the substrate

TABLE III

Effect of Griseofulvin and Sodium Azide on Oxygen Uptake (Q_{O_2}) by Botrytis allii in Weindling Medium at 25° C.

Expt. No.	Control.	Q_{O_2} griseofulvin.			Azide.
		I μg./ml.	10 μg./ml.	100 μg./ml.	100 μg./ml.
10	11·7, 11·5	—	11·5, 11·2	—	—
11	11·4, 10·4	9·8, 11·0	11·1, 10·9	—	—
13	11·3, 10·4	—	10·6	10·0	1·2
14 ¹	17·6	—	—	17·5	1·7

¹ Mycelium from cultures on medium supplemented with yeast.

(3 ml. in each case) before beginning. Oxygen uptake is expressed as Q_{O_2} (μl. oxygen taken up per hour per mg. of dry weight). Azide, a well-known respiratory inhibitor used here as a standard of comparison, reduced oxygen uptake severely at 100 μg./ml. This latter concentration is near to the minimum concentration needed to inhibit germination of *Botrytis allii* conidia. Griseofulvin, on the contrary, had no significant effects at any of the concentrations tested.

This is confirmed by the results of another experiment shown in Table IV. In this case respiration of a mycelial suspension in water was measured in

TABLE IV

Effect of Griseofulvin and Sodium Azide on Oxygen Uptake (Q_{O_2}) by a Suspension of Botrytis allii Mycelium in Water at 25° C.

	Substances added from side arm.		
	(a) Water (control).	(b) Griseofulvin (10 μg./ml.).	(c) Azide (100 μg./ml.).
Q_{O_2} before tipping side arm .	14·8	14·7, 14·0	13·8
Q_{O_2} after tipping side arm .	15·2	16·8, 13·1	2·0

each of four manometers for 30 minutes; griseofulvin was then added to two of the flasks from side arms to give a final concentration of 10 μg./ml., azide was added to a third to give a final concentration of 100 μg./ml., and to the fourth (control) flask an equivalent quantity of water was added, and respiration was measured for a further 30 minutes. It will be seen that whereas azide severely reduced oxygen uptake, griseofulvin had no effect.

It has been shown, then, that at concentrations at which griseofulvin has marked morphogenetic effects it does not affect oxygen uptake. In the case of the experiment described in Table IV a morphological response was obvious 10 hours after the experiment commenced and a further measurement of oxygen uptake showed it to be still unaffected.

(d) Comparison with the stunting effect of some other substances on *Botrytis allii* hyphae

The effect of a number of other substances, mostly synthetic, on the germination of *Botrytis allii* spores has been compared with the effect of griseofulvin. The substances chosen for test fell into the following groups:

Cyclic acids and esters: Vincent (1947, 1947a) has reported that a number of simple aromatic acids and their esters produce effects similar to those of griseofulvin, though higher concentrations are needed. Results obtained with several of the substances mentioned by Vincent, related substances, and a number of cyclic acids of fungal origin are given in Table V.

TABLE V

Effect of Miscellaneous Cyclic Acids and their Esters, including Acids produced by Fungi, on Germination and Early Growth of *Botrytis allii*

Substance.	Concentrations ($\mu\text{g.}/\text{ml.}$).			
	1,000.	100.	10.	1.
benzoic acid	—	—	100S	100
carlic acid	100SS	100	100	100
carolic acid	—	100SS	100	100
trans-cinnamic acid	—	—	100	100
dehydrocarolic acid	—	50SS	100	100
p-hydroxybenzoic acid	50SS	100S	100	100
methyl p-hydroxybenzoate	—	50SS	100	100
mycophenolic acid	—	100SS	100SS	100S
phenol	100S	100	100	100
salicylic acid	—	—	100	100
terrestric acid	—	—	90SS	100
griseofulvin	—	100SS	100SS	100SS

(Note: — = no germination; 50 = 50% germination, &c.; SS = severe stunting of germ-tubes; S = less severe, but noticeable stunting.)

Heteroauxin and analogous substances: for reasons discussed more fully in Section VI it was considered that the action of griseofulvin on fungus hyphae was analogous to some of the effects of indolylacetic acid (heteroauxin) and related substances on the growth of root-hairs, roots, and other angiosperm tissues. Results with a number of such substances are given in Table VI.

Amino-acids: Audus and Quastel (1947a) have recently reported inhibitory activity of amino-acids on germination of seeds. It was considered desirable by analogy to investigate the effect of amino-acids on germination of *B. allii* conidia (Table VII).

In all cases serial dilutions (1 in 10) of solutions or dispersions of the substances in Weindling medium at pH 3.5 were inoculated with conidia of *B. allii* and their germination followed at 25° C.

Considering first Table V, we see that most of the substances tested were mildly fungistatic, preventing germination at high concentrations. p-Hydroxybenzoic acid and its methyl ester had a pronounced stunting effect in sub-fungistatic doses, as noted by Vincent. Not only were relatively high

TABLE VI

Effect of Indolylacetic Acid and Analogous Substances on Germination and Early Growth of Botrytis allii

Substance.	Concentration ($\mu\text{g./ml.}$).			
	1,000.	100.	10.	1.
2-chlorophenoxyacetic acid . . .	—	80SS	100	100
3-chlorophenoxyacetic acid . . .	—	—	100S	100
4-chlorophenoxyacetic acid . . .	—	30SS	100S	100
α -(2-chlorophenoxy) propionic acid	—	100SS	100	100
α -(3-chlorophenoxy) propionic acid	—	100SS	100	100
2,4-dichlorophenoxyacetic acid . .	—	50SS	100	100
2,5-dichlorophenoxyacetic acid . .	—	100SS	100	100
3-indolylacetic acid . . .	100SS	100S	100	100
methyl 3-indolylacetate . . .	—	100SS	100	100
γ -3-indolylbutyric acid . . .	50SS	100S	100	100
methyl γ -3-indolylbutyrate . . .	—	50SS	100	100
2-methyl-4-chlorophenoxyacetic acid	—	—	100S	100
α -naphthoxyacetic acid . . .	—	50SS	100	100
ethyl α -naphthoxyacetate . . .	—	100SS	100	100
β -naphthoxyacetic acid . . .	—	—	100S	100
ethyl β -naphthoxyacetate . . .	100SS	100SS	100S	100
methyl β -naphthoxyacetate . . .	50SS	100SS	100SS	100
isopropyl β -naphthoxyacetate . . .	100SS	100SS	100S	100
β -naphthoxyacetoneitrile . . .	—	30SS	100	100
α -naphthylacetic acid . . .	—	—	100	100
β -(α -quinolyl) acrylic acid . . .	—	—	100	100
colchicine . . .	—	100	100	100
coumarin . . .	100	100	100	100
isopropyl phenylcarbamate . . .	—	100	100	100
griseofulvin . . .	—	100SS	100SS	100SS

(Note: — = no germination; 50 = 50% germination, &c.; SS = severe stunting of germ tubes; S = less severe, but noticeable stunting.)

concentrations needed but the stunting was in appearance quite distinct from that produced by griseofulvin. The stunting only involved a shortening of branches and internodes and more cell division to a given length of hypha; there was no distortion, spiral curling, swelling, or spatulate ends to the hyphae. A similar effect was produced by a number of the other substances tested, the most effective being mycophenolic acid. It is proposed to refer to this type of stunting, which is far less specific than that produced by griseofulvin, as 'cyclic acid stunting'.

Many of the heteroauxin analogues also were mildly fungistatic and many, too, produced typical 'cyclic acid stunting'. The most effective was methyl β -naphthoxyacetate. Three other substances—colchicine, coumarin, and isopropyl phenylcarbamate—were included because of their inhibitory activity on various phases of angiosperm growth, had no effect on *Botrytis allii* in relatively high concentrations.

Of the amino-acids, *p*-aminobenzoic acid, *o*-aminobenzoic acid (anthranilic acid), β -phenylalanine, and tyrosine produced 'cyclic acid stunting'. The toxicity of *p*-aminobenzoic acid to fungi has been recorded by Cavill and Vincent (1945, 1948). A distinct type of stunting, more reminiscent of the

TABLE VII

Effect of Amino-acids on Germination and Early Growth of Botrytis allii

Amino-acid.	Concentration ($\mu\text{g./ml.}$).			
	1,000.	100.	10.	1.
<i>dl</i> -alanine	100	100	100	100
β -alanine	80SS	100SS	100S	100
<i>o</i> -aminobenzoic acid	—	50SS	100S	100
<i>p</i> -aminobenzoic acid	50SS	100SS	100	100
<i>l</i> -arginine monohydrochloride	100	100	100	100
asparagine	100	100	100	100
<i>l</i> -aspartic acid	100	100	100	100
<i>l</i> -cystine	100	100	100	100
<i>l</i> -glutamic acid	100	100	100	100
glycine	100SS	100S	100	100
<i>l</i> -histidine	100	100	100	100
<i>l</i> -leucine	100	100	100	100
β -phenylalanine	100S	100	100	100
<i>l</i> -proline	100	100	100	100
<i>dl</i> -serine	100	100	100	100
<i>l</i> -tryptophan	100	100	100	100
<i>l</i> -tyrosine	100S	100	100	100
griseofulvin		100SS	100SS	100SS

(Note: — = no germination; 50 = 50% germination, &c.; SS = severe stunting of germ-tubes; S = less severe, but noticeable stunting.)

effect of griseofulvin in that germ-tubes with abnormal swellings and spatulate tips were formed, was produced by β -alanine and glycine. Relatively high concentrations of these compounds were needed.

It is concluded from these results that the stunting produced by griseofulvin is of a highly specific nature; a final judgement must await examination of the biological properties of analogous and degradation products.

IV. EFFECTS OF GRISEOFULVIN ON BACTERIA AND ACTINOMYCETES

Bacteria. Broth was made up containing 100 $\mu\text{g./ml.}$ griseofulvin, inoculated with *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli*, and *Bacillus subtilis*. No inhibition of growth was observed and all growth forms were normal. Similarly, griseofulvin had no effect on the growth of these bacteria on agar media.

Actinomycetes. *Actinomyces albosporeus* (NCTC 1578), *A. chromogenes* (NCTC 1569), *A. gardneri* (NCTC 6531), and *A. madurae* (NCTC 1070) were grown on prune agar containing griseofulvin at concentrations of 1, 5, 10, and 20 $\mu\text{g./ml.}$ No inhibition of growth was observed and colonial and cell morphology appeared to be normal.

V. TOXICITY OF GRISEOFULVIN TO ANGIOSPERM SEEDS

The effect of griseofulvin on the germination of wheat, clover, and mustard seeds is shown in Table VIII. Coumarin and β -indolylacetic acid were included in the experiment as standards of comparison. The seeds were incubated for 4 days at 25° C. on agar containing the substances to be tested.

Growth of the wheat was measured by fresh weight of roots, of the other seeds by measurement of length of main root. Results are presented in Table VIII.

TABLE VIII
Toxicity of Griseofulvin, β -indolylacetic Acid, and Coumarin to Angiosperm Seeds

Compound.	Concentration (μ g./ml.).	Germination %.			Root growth. ¹		
		Clover.	Mustard.	Wheat.	Clover.	Mustard.	Wheat.
coumarin . . .	1	100	91	100	55	59	100
	5	97	71	100	55	41	73
	25	89	61	100	36	23	8
griseofulvin . . .	1	97	88	100	82	66	96
	5	96	86	100	67	51	80
	25	100	61	100	48	23	12
β -indolylacetic acid	1	90	86	100	31	11	86
	5	91	89	100	22	11	68
	25	76	64	100	8	7	7

¹ As per cent. of control, only germinated seeds considered.

It will be seen that griseofulvin is appreciably toxic to seeds; it is of the same general order of activity as coumarin (Audus and Quastel, 1947), but less active than β -indolylacetic acid on clover and mustard.

Griseofulvin produced none of the formative effects (swelling of hypocotyl and distortion of root-hairs) characteristic of β -indolylacetic acid and is therefore probably quite distinct in its mode of action.

VI. DISCUSSION

The biological activity of griseofulvin may be summarized as follows: (a) it produces marked morphogenetic disturbances of many fungi without any simultaneous effect on respiration or glycolysis and, in spite of the unique response as seen in detail, its overall effect on the growth rate of the fungus most studied (*Botrytis allii*) does not differ in its general dose/response relationships from that of simpler toxicants such as phenol; (b) it has no apparent effect on bacteria, actinomycetes, or on certain groups of fungi (Oomycetes and yeasts); (c) it inhibits the germination of seeds of higher plants and retards root extension.

The effect on filamentous fungi is most specific and it is this aspect of the activity of griseofulvin that is considered most fully below.

The nature of the effect on hyphal growth

Little is known concerning the mechanism of hyphal extension in fungi. It is not in general possible to distinguish between cell multiplication and cell elongation as it is when considering the growth of an angiosperm root or shoot and it cannot be said with certainty whether the hypha increases in length at

or near the tip only or in the older, vacuolate, parts of the hypha also. It seems most probable that growth is localized to a region near the tip, though branches may be initiated at some distance behind this. Once the hypha is formed growth is entirely longitudinal; the hypha has usually a characteristic thickness which is constant throughout a given colony. One envisages, therefore, that growth takes place by deposition of new cell-wall material at or just behind the hyphal tip or, more probably, a softening of the cell-wall at the tip resulting in a stretching of the cell-wall as a result of internal pressure, followed by intussusception of new cell-wall material. In either case the structural characteristics of the cell-wall appear to favour longitudinal rather than transverse stretching.

With such a picture in mind, it is instructive to reconsider the effects of griseofulvin on sensitive fungi.

(i) Very low concentrations ($0.2-0.1 \mu\text{g./ml.}$) cause a regular spiral curling of the hyphae; in this connexion it should be noted that most hyphae show an inconspicuous, highly attenuated sinuosity, that it has been observed that growth of certain hyphae, notably the sporangiophores of *Phycomyces*, is spiral, the growing hyphal tip consequently rotating (Oort, 1931; Oort and Roelofsen, 1932), and that this has been correlated with the tangential arrangement of the structural molecules (chitin) in the sporangiophore wall (Heyn, 1936).

(ii) Somewhat higher concentrations ($c. 1 \mu\text{g./ml.}$) cause excessive branching with much shortened internodes, abnormally thick hyphae with unusual swellings and distortions of various kinds, and with spatulate hyphal tips.

(iii) Higher concentrations ($10-20 \mu\text{g./ml.}$) cause a severe stunting; the germ-tubes produced are swollen and misshapen and there is a complete failure to develop the hyphal form.

All these responses may be characterized as 'cell-wall effects'. In the lower doses ($c. 0.1 \mu\text{g./ml.}$) the organization of the growing tip does not appear to be disturbed but the normal inconspicuous sinuosity, possibly a gross reflection of a tangential fine-structure, is exaggerated; if, as Heyn (1936) suggests, the spiral growth of *Phycomyces* is due to slipping along the longitudinal planes of the orientated structural molecules, and if this situation is general in fungal hyphae, the action of very low concentrations of griseofulvin might be regarded as controlling the amount of such slipping along crystal planes or as affecting the orientation of newly deposited structural molecules. In the medium doses ($1 \mu\text{g./ml.}$) the organization of the growing tip is disturbed and the effects produced suggest that 'weak spots' in the cell-wall appear in a disorganized manner, resulting in excessive branching, and that the resistance of the cell-wall to radial stretching is greatly reduced either locally or over the whole surface of the hypha. In the higher doses ($10-20 \mu\text{g./ml.}$) a localized growing-point seems to be completely lost and stretching of the cell-wall is almost unlocalized. The 'giant cells' produced by *Aspergillus niger* in the presence of pentachlorophenol (Armitage and Verdcourt, 1948) are very similar to this stage. Thus all these responses are explicable in terms of alteration

in the physical properties of the cell-wall and the transformation of a system of organized local change in plasticity to one of disorganized and more general plastic changes.

Support for this view is afforded by a study of disturbances in the development of angiosperm root-hairs, organs morphologically similar to fungal hyphae. Cormack (1935) has shown that the cell-wall of root-hairs is dual in nature, containing a cellulose layer and a pectic layer, continuous with corresponding layers in the epidermal cell. Rigidity in healthy root-hairs is conferred not by the cellulose layer but by the pectic layer which is mainly composed of calcium pectate in the longitudinal walls of the root-hair, though in the growing tip a lower proportion of the pectic acid is combined with calcium and the wall is correspondingly more plastic. Under conditions of calcium starvation abnormalities in development result. If calcium deficiency is partial, distortions and branching result, very reminiscent of the effect of griseofulvin on fungus hyphae. Cormack regards the effect to be due to failure to deposit the rigid calcium pectate layer evenly, the internal osmotic pressure thus causing swelling and extrusion of branches from the weak spots. In conditions approaching absolute calcium deficiency, the whole cell-wall remains evenly plastic, instead of the plastic zone being localized at a growing tip, and consequently no root-hairs are formed and the cells expand more or less symmetrically. This effect is reminiscent of that produced by high concentrations of griseofulvin. This disturbance in development of root-hairs, highly analogous to the disturbance in hyphal development produced by griseofulvin, has been shown with little doubt to be associated with disturbances in the plasticity of the cell-wall.

A second analogy can be seen in the effect of β -indolylacetic acid (hetero-auxin) on root-hairs. The distortion of leguminous root-hairs caused by infecting root-nodule bacteria (Hiltner, 1900; McCoy, 1932) has been shown to be due to secretion of β -indolylacetic acid by the bacteria. Solutions of indolylacetic acid and some related substances, in very low concentrations, produce a spiral curling, branching, distortion, and spatulate swelling of the root-hair tips (Chen, 1938; Nutman, Thornton, and Quastel, 1945). This effect is similar in appearance to that of griseofulvin on fungal hyphae and is regarded as a 'cell-wall effect'.

These observations all suggest that an understanding of the action of griseofulvin on fungi will involve an investigation of the nature, formation, and extension of the hyphal cell-wall. Consideration of the differential response of various major groups of fungi leads to the same conclusion.

The differential response of major groups of fungi

In Section III it was shown that in general Basidiomycetes, Ascomycetes, Fungi Imperfecti, and Zygomycetes (Mucorales) are sensitive to griseofulvin, responding by the morphogenetic abnormalities described, whereas the Oomycetes (Saprolegniales and Peronosporales) and certain yeasts did not respond in this manner. The reduction of growth rate of *Achlya* is regarded

as being of no significance in this connexion in view of the complete absence of any morphogenetic response.

Ignoring for the moment the yeasts, which will be considered later, it may be said that the broad distinction in response to griseofulvin, between the Oomycetes on the one hand and the Ascomycetes, Basidiomycetes, and Fungi Imperfecti on the other, also follows a broad distinction in the chemical nature of the cell-wall. There is much evidence that most Oomycetes (*all* Saprolegniales and Peronosporales, into which orders the Oomycete fungi tested fell) have cellulose cell-walls and that Ascomycetes, Basidiomycetes, and Fungi Imperfecti have chitin cell-walls. If this evidence is correct, the distinction in cell-wall composition between griseofulvin-sensitive and griseofulvin-tolerant fungi is probably significant, particularly since the griseofulvin effect has the appearance of a cell-wall effect. The data bearing on the composition of fungal cell-walls will therefore be briefly reviewed.

Cellulose is easily and reliably demonstrated by the purple or blue colour reaction with chlor-zinc-iodide. Using this method cellulose cell-walls have been found to be characteristic of the Lagenidiales, Saprolegniales, and Peronosporales (the classification proposed by Martin (1941) has been used in this discussion) among the mycelial Oomycetes (van Wisselingh, 1897; Petersen, 1909; von Wettstein, 1921; Nabel, 1939). They are also found among the Archimycetes in the Woroninaceae. In all these cases the possession of cellulose cell-walls is correlated with the production of equally biflagellate zoospores and is obviously of considerable phylogenetic and systematic significance. (For discussion of the nature of the fungus cell-wall in relation to phylogeny see von Wettstein, 1921; Scherffel, 1925; Harder, 1937, 1939; Nabel, 1939; Karling, 1942; Sparrow, 1943; Ulrich, 1943.)

Surveys made by van Wisselingh (1897), von Wettstein (1921), and Nabel (1939) have shown that the cell-walls give no colour reaction with chlor-zinc-iodide, other than a pale yellow stain, in the Basidiomycetes, Ascomycetes, Fungi Imperfecti, Zygomycetes, and, among the Oomycetes, in the Monoblepharidales and Mycochytridiales, and in all the Archimycetes except the family Woroninaceae. Further, in all these cases the cell-walls give a reddish-purple colour with van Wisselingh's chitosan test which is regarded as specific for chitin; animal chitin gives a similar colour reaction. The chemical identity of fungus chitin with animal chitin was first demonstrated beyond doubt by Diehl and van Iterson (1935) using the sporangiophore of *Phycomyces blakesleeana*. Identity of crystal structure, using X-ray techniques, has been demonstrated by van Iterson, Meyer, and Lotmar (1936) and by Heyn (1936). Using macrochemical methods Schmidt (1936) has shown chemically defined chitin to be present in the Basidiomycetes, Ascomycetes, Fungi Imperfecti, and Zygomycetes but not in the Oomycetes, several genera and species being used in each case. There seems little doubt therefore that the surveys based on the microchemical chitosan test are substantially correct.

Returning now to the results presented in Table II it will be seen that all those forms which gave a morphogenetic response to griseofulvin have chitin

cell-walls. Of those that gave no response the Oomycetes were shown to give a positive cellulose reaction by the chlor-zinc-iodide test, leaving only the two yeasts, *Saccharomyces cerevisiae* and *Torulopsis utilis*, as apparent exceptions. Schmidt (1936) and Zechmeister and Toth (1934, 1936) have confirmed the suggestion of van Wisselingh (1897) that the cell-wall of the yeasts is based on a polysaccharide distinct from either chitin or cellulose. It thus appears true to say that, on the basis of the limited number of observations made, griseofulvin produces a morphogenetic disturbance in those fungi possessing chitin cell-walls. If griseofulvin is specific to chitin cell-walled organisms, its complete lack of toxicity to bacteria or actinomycetes is explained, the cell-walls of these organisms having a different structural basis.

It would be of interest to examine the effect of griseofulvin on those members of the Archimycetes and Oomycetes said to have chitin cell-walls. This presents some difficulty as many of these forms are obligate parasites or otherwise difficult to culture independently. Preliminary experiments with *Allomyces javanicus* Kniep have indicated that growth is completely prevented by 5 $\mu\text{g.}/\text{ml.}$ of griseofulvin. In this respect *Allomyces* differs from the cellulose cell-walled Oomycetes tested, but, on the other hand, no morphogenetic response was seen.

Growth regulation in fungi

Morphogenesis in angiosperms is largely controlled by the auxin system. Application of substances with auxin-activity to the plant results in morphogenetic abnormalities. Examples are the root-hair distortion already discussed, production of epinastic curvatures as a result of unilateral cell extension, stimulation of production of adventitious roots on shoots, prevention of seed germination, and inhibition of root growth. Many of these effects are believed to be due to direct or indirect effects on the plasticity of the cell-wall (Went and Thimann, 1945). It is characteristic that the response to auxin may vary qualitatively with quantitative variations in dose. Morphogenetic disturbances may also be produced by substances which affect the internal auxin system in the plant, e.g. the effect of ethylene on development of angiosperm shoots.

There is much in the observed effects of griseofulvin on fungi which suggests that its mode of action may be concerned with a growth-regulating mechanism. It is clear that to produce so highly organized a structure as the Basidiomycete sporophore some growth-regulating mechanism must exist. Normal hyphal development, involving regular branching and dominance of main growing-points over secondary branches, suggests that there is, even on this simple plane, growth regulation by a system analogous to the auxin system. The effect of moderate doses (*c.* 1 $\mu\text{g.}/\text{ml.}$) of griseofulvin on developing *Botrytis allii* hyphae can be characterized as a lack of apical dominance, analogous in appearance to the effect of ethylene on potato shoots illustrated by Borgström (1939). Nothing is known of any such growth-regulating mechanism in fungi. Skoog (1947) has remarked that 'in organisms built on

different structural bases, such as the fungi and bacteria, auxin may be essential, as judged by its universal presence, but it does not exert a corresponding morphogenetic function'. This is confirmed by results given in Section III. On the other hand, the effect of griseofulvin on higher plants showed no real analogy to the effect of auxins, as none of the typical effects of auxins—root-hair curling and hypocotyl hypertrophy—were produced. It seems possible, however, that griseofulvin is either itself a growth-regulating substance for chitin-walled fungi or that it specifically interferes with a growth-regulating system in such fungi.

VII. SUMMARY

Griseofulvin ($C_{17}H_{17}O_6Cl$), a metabolic product of several species of *Penicillium*, is shown to influence profoundly the morphogenesis of many fungi. The effects, produced by concentrations in the range $0.1-10.0 \mu g./ml.$, include severe stunting, excessive branching, abnormal swelling and distortion of hyphae, and production of spirally twisted hyphae. These effects were transitory, transplants from a griseofulvin-containing medium to a normal medium producing normal growth.

Of fifty-one species of fungi examined, all Basidiomycetes, Ascomycetes, Fungi Imperfecti, and Zygomycetes, with the exception of two yeasts, were sensitive to griseofulvin. None of the Oomycetes examined (all falling into the orders Saprolegniales and Peronosporales) were affected by griseofulvin.

The effect of griseofulvin on a sensitive fungus (*Botrytis allii*) was examined in more detail. It was found that its depressing effect on growth rate, in spite of the specificity of the response as seen in detail, was comparable in its dose/response relationships to that produced by simple toxicants such as phenol. No effect on oxygen uptake could be demonstrated in concentrations capable of markedly affecting growth rate.

A number of other substances were shown to produce marked stunting of *Botrytis allii* germ-tubes in concentrations well below those inhibiting germination. These include a number of heteroauxin analogues and other cyclic acids and their esters; all these substances produced a non-specific stunting ('cyclic acid stunting') distinct from that produced by griseofulvin. Among the most active of these substances was another mould product, mycophenolic acid. Two amino-acids, glycine and β -alanine, in relatively high concentrations (*c.* $100 \mu g./ml.$) produced a morphogenetic effect more truly like that of griseofulvin.

Griseofulvin had no effect on growth or morphology of any of a range of Actinomycetes and bacteria examined. It was toxic to angiosperm seeds; root extension of wheat, mustard, and clover was much retarded in seeds germinating on agar containing $25 \mu g./ml.$ griseofulvin.

Reasons are given for supposing that the effect of griseofulvin on sensitive fungi is directly or indirectly on the cell-wall or on the process of extension of the cell-wall. In this connexion it is significant that all those fungi which are sensitive to griseofulvin have chitin cell-walls and all those unaffected

(Peronosporales, Saprolegniales, and yeasts) have cellulose or other non-chitinous cell-walls. It is suggested that fungi have a growth-regulating mechanism analogous to the auxin system in higher plants, but based on a distinct chemical system, and that the mode of action of griseofulvin is concerned with its effect on such a growth-regulating system.

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Cation Adsorption by Brown Algae: The Mode of Occurrence of Alginic Acid

BY

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THE interaction of simple ions with plant cells has been frequently studied and it has been found that in some cases cations and anions are both absorbed in the vacuoles (for references see Steward, 1935; Hoagland, 1915 and 1937; Krogh, 1946), while under different conditions the readjustment of the salt content occurs mainly by ion exchange (see, for instance, Genevois, 1930; Camlong and Genevois, 1930; Genaud and Genevois, 1930; Lundegardh, 1939). The experiments now to be described belong to the latter type; they were done both in a static system and in a continuous flow, and, so far as I am aware, this is the first time that the ionic adsorption by a plant material has been systematically studied under conditions comparable to those prevailing in inorganic chromatographic adsorption analysis.

The measurements were made with brown algae, which contain a number of water-insoluble acidic substances including alginic acid, this high polymer having received much attention because it can be converted into useful fibrous or gelatinous materials. Some of the present adsorption measurements are of interest with regard to the question as to whether brown algae contain the alginic acid in the free state or rather in the form of alginates, while other observations indicate optimum conditions for the uptake of a particular cation by one type of algal material. Under continuous flow conditions only cations appear to be adsorbed, but in static systems, where slow reactions are of greater importance for the over-all effects, an anion adsorption could also be detected.

EXPERIMENTAL PART AND RESULTS

The adsorption by the whole plant material was measured and no separate sap analyses were made. Preliminary tests showed that there was no measurable influence of illumination or of the rate of oxygen supply and, therefore, the majority of the runs were done without bubbling oxygen through the reaction mixture. The freshly collected air-dry algae were broken up in a porcelain ball mill and fractions of the required size distribution were separated by sieves. If nothing else is mentioned, particles taken from the fronds and of a size between 12 and 25 mesh to the inch were used, all operations being done at temperatures between 17° and 21°. After washing the broken-up algae thoroughly for 12 hours the great majority of the cells appeared to be dead.

Some of the figures in the following tables relate to 1 kg. dry algal material, these dry weights being estimated with aliquots of the water-washed tissue, prior to the extraction with 1 N hydrochloric acid. This latter operation was done as specified in an earlier paper (Wassermann, 1948).

Sodium, potassium, calcium, barium, magnesium, aluminium, chromium, iron, manganese, copper, lead, bismuth, and silver were determined as uranyl-magnesium-triple acetate, cobaltinitrite, oxalate, sulphate, pyrophosphate, oxide, sulphide, and chloride. Calcium, magnesium, copper and silver were also estimated respectively with palmitate (see Suter, 1937), thiosulphate, and thiocyanate, and iron and manganese colorimetrically with thiocyanate and formaldoximehydrochloride. Ammonia, chloride, and aniline were determined according to Kjeldahl, Volhard, and colorimetrically with calcium hypochlorite. The acidities were measured with a valve pH meter and glass electrodes.

The principal metals in the tissues of water-washed *Ascophyllum nodosum*, *Fucus vesiculosus*, *Laminaria digitata*, and *Laminaria saccharina* are sodium, potassium, calcium, magnesium, aluminium, and traces of iron. These were determined both before and after the algae has been extracted with 1 N hydrochloric acid. Alginic acid or alginates (*a*), other water-insoluble acids or their salts (*b*), and esters (*c*) are constituents which are also of interest. The constitution of (*b*) and (*c*) is unknown; as the water extracted or hydrochloric acid treated materials contain a relatively large amount of nitrogen it is feasible that either (*b*) or the acid component of (*c*) is an amino-acid or a peptide. The alcoholic component of (*c*) may be the alginic acid, by virtue of its alcoholic hydroxyl groups. The results of the various analyses made with *Ascophyllum nodosum*, collected in September 1945, are shown in Table I.

In estimating the figure in the seventh line (Tollens-Lefevre method) the algal material equivalent to a dry weight of 2.86 g. was refluxed with 100 ml. 15 per cent. hydrochloric acid; the following results were obtained:

Time (hours)	1½	3¼	5	21	∞
CO ₂ as percentage of dry weight	3.85	6.15	7.00	8.73	8.75

a blank experiment showing that a correction of -0.35 per cent. had to be applied.¹ The hydrochloric acid extracted algal material, equivalent to a dry weight of 19.1 g., was shaken for 24 hours with 2 litres 0.1 N sodium hydroxide, thereby obtaining a solution of sodium alginate. After separating and washing the residue, the alginic acid precipitated with hydrochloric acid was spun down and aliquot well-washed portions were analysed by carrying out carbon dioxide determinations according to the Tollens-Lefevre method and alkalimetric titrations. It was found that 0.036 g. equivalent alginic acid had been precipitated, which leads to the figure in the eighth line (column 3) of Table I. In determining (*a*)+(*b*) (tenth line of Table I) a titration method similar to that described by Wassermann (1948*a*) was used. The hydrochloric acid extracted and water-washed algal material equivalent to a dry weight of 1.50 g. was placed in 50 ml. water, containing phenolphthalein as

¹ Similar tests were done to deduce the figure in the eighth line of the first column.

TABLE I

Exchange of Metals against Hydrogen Ions in the Treatment of Ascophyllum with 1 N Hydrochloric Acid; all figures are g.-equivalents/kg. dry algal material

	Determined in H ₂ O washed alga.	Determined in HCl extracted and subsequently H ₂ O washed alga.	Deduced from analyses made in filtrate and washings as obtained during the HCl ex- traction of the alga.
Sodium	0.82 ± 0.02	0.16 ± 0.04	0.66 ± 0.024
Potassium . . .	0.45 ± 0.02	0.01 ± 0.04	0.44 ± 0.024
Calcium	0.77 ± 0.02	0.12 ± 0.04	0.65 ± 0.024
Magnesium . . .	0.20 ± 0.01	0.01 ± 0.02	0.19 ± 0.012
Aluminium and traces of iron	0.8 ± 0.1	0.2 ± 0.05	0.6 ± 0.15
Sum of principal metals	3.0 ± 0.2	0.5 ± 0.2	2.5 ± 0.4
Alginic acid (a) or its derivatives . . .	1.9 ± 0.1*	1.9 ± 0.1*	
Other water-insoluble acids (b)	—	0.9 ± 0.3	—
Acids (a) + (b) . . .	—	2.8 ± 0.2	—
Esters (c)	—	2.3 ± 0.5	—
Hydrogen ions absorbed by water-washed alga	—	—	3.0 ± 0.3
Chloride ions absorbed by water-washed alga	—	—	1.0 ± 0.1
Nitrogen	1.6 ± 0.1	1.6 ± 0.1	—

* Deduced from the results of analyses made according to the Tollens-Lefevre method.

† Deduced from yield of alginic acid as obtained during its preparative isolation.

indicator, the suspension being rapidly stirred and kept free of atmospheric carbon dioxide, while 0.172 N barium hydroxide was added in portions of 1.0 ml. It was observed that on addition of the first 22 portions, the time, t , which is required until the pink coloration of the indicator disappears was in each case smaller than 15 minutes (first stage of the titration). Similar values were observed in control tests, in which the barium hydroxide solution was added to a stirred suspension of alginic acid. After further addition of 1.0 ml. portions of 0.172 N barium hydroxide to the algal material the time t rose abruptly to about 50 minutes (second stage of the titration). The (a) + (b) value referred to above was estimated by taking into account that 1.50 g. algal material consumed during the first stage of the titration 3.8×10^{-3} g.-equivalent barium hydroxide. In another experiment the hydrochloric acid extracted algal material equivalent to a dry weight of 1.92 g. was suspended for 20 hours in 150 ml. 0.172 N barium hydroxide. The solution was then filtered off and the residue was slowly washed with 1 l. water, atmospheric carbon dioxide being excluded. Titrations and barium determinations made in the joint filtrate and washings and in the residue showed that the alga had consumed 9.7×10^{-3} g.-equivalent barium hydroxide; it follows that 1 kg. of the dry material would have consumed 5.1 g.-equivalent and if 2.8 g.-equivalent are deducted the content of esters designated in Table I by (c) is obtained. An indirect method of estimating (a) + (b) was as follows. After the water-washed

algal material had been extracted with a known quantity of 1 N hydrochloric acid, the joined filtrates and washings were titrated to $\text{pH} = 7$. This simple method of analysis (leading to the figure in line 12, last column of Table I) is applicable, although the alga absorbs not only hydrogen ions but also chloride, because the acid hydrolysis of (c) is negligibly slow at room temperature and because pH measurements indicate that the acids¹ which are neutralized during the alkalimetric titrations are practically completely dissociated. The only complication is due to the dissolution of aluminium salts which hydrolysed; the apparent hydrogen-ion adsorption, as deduced from the uncorrected titration results, is too small, therefore, but it could be shown that the error is not larger than 10 per cent. The other algae mentioned above were similarly analysed and the results are compatible with the same stoicheometric relationships as those to be deduced from Table I and discussed on page 85.

In estimating the metal ion adsorption, under static conditions, by water-washed *Ascophyllum nodosum* (I), *Laminaria digitata* (III), *Laminaria saccharina* (V), and by the same species after extraction with hydrochloric acid and subsequent washing with water (II, IV, VI), the materials were suspended in solutions of the salts listed in the second line of Table II, under concentration and volume conditions specified in the third and fourth lines.

The pH values of the solutions (see sixth and seventh lines) were measured at zero time and after the times indicated in the fifth line. After these measurements the algae were filtered off, slowly washed with water, and finally the combined filtrates and washings were analysed, thus enabling a computation of the figures in the last four lines. The metal and hydrogen ions release values α and β are defined by:

$$\alpha = \frac{\text{Number of equivalents of released ions of principal metals}}{1 \text{ equivalent of adsorbed metal ion}}$$

$$\beta = \frac{\text{Number of equivalents of released hydrogen ions}}{1 \text{ equivalent of adsorbed metal ion}}.$$

The figures in the last line of columns 2, 4, 6, 8, 10, 12, 14, and 16 are metal ion release values, α , which relate to the algae I, III, and V, while the figures in the other columns are hydrogen ion release values, β , and relate to the materials II, IV, and VI.

Control experiments were made which showed that the metal ions released by algae I, III, or V are not due to traces of sea-water being kept in the interstitial spaces of the cell tissue, and that the release of hydrogen ions from algae II, IV, and VI cannot be due to incompletely removed hydrochloric acid. The cation adsorption was in all cases accompanied by an anion adsorption. No tests were made to find out whether an anion exchange occurred, and the β values were calculated on the assumption that the released acid is practically completely dissociated; this could only be proved, however, in the experiments with the chlorides and with silver nitrate. Potassium, sodium,

¹ The whole acidity is not necessarily due to hydrochloric acid; other acids may have been liberated as a result of anion exchange.

TABLE II

Ionic Adsorption under Static Conditions by Water-washed Ascophyllum, Laminaria digitata, and L. saccharina (I, III, V), and by the same Species after Treatment with Hydrochloric Acid (II, IV, VI)

	Ascophyllum.										L. digitata.			L. saccharina.		
	I	II	I	II	I	II	I	II	I	II	III	IV	V	VI	V	VI
Alga used	NaCl	KCl	CaCl ₂	MgCl ₂	CuSO ₄	AgNO ₃					CaCl ₂	CaCl ₂	CaCl ₂			
Alga placed in solution of	0.0075	0.0075	0.0119	0.0100	0.0192	0.0100					0.0119	0.0119	0.0119			
Conc. of solution (g.-equivalent/l.)	50	50	117	50	105	110					66	66	66			
ml. solution per 1 g. dry alga*	24	24	24	48	90	48					24	24	24			
Time of absorption (hrs.)	6.0	6.3	5.5	5.8	6.0	6.0					5.5	6.0	5.5			
pH of solution { zero time	6.0	6.4	5.5	5.8	4.3	4.3					5.9	2.4	5.5			
end of test	6.0	2.7	2.8	2.9	4.5	2.7					2.4	2.4	5.5			
Per cent. cation adsorbed	10	67	56	73	50	88	72	30	64	54	50	45				
g.-equivalent of cation adsorbed per kg. dry alga	0.04	0.3	0.2	0.3	0.6	0.5	1.6	1.6	0.8	0.3	0.5	0.4	0.4	0.4		
Principal cations released from alga	K ⁺	H ⁺	Na ⁺	H ⁺	Na ⁺ K ⁺	H ⁺	Na ⁺ K ⁺	Ca ⁺⁺	Mg ⁺⁺							

* The experiments were made with an amount of algal material equivalent to a dry weight of at least 4 g.

Metal ion release value (α) and hydrogen ion release value (β)

0.9 0.5 0.9 0.5 0.9 0.8 1.0 0.6 1.0 0.2 1.0 1.0 0.7 1.0 0.6

calcium, magnesium, and aluminium ions could be detected in the final filtrates and washings of most of the experiments, but except where indicated in Table II, the quantity was negligible compared to that of the cation adsorbed by the algae. No tests were made, however, to find out whether traces of organic bases are released. The calcium ion adsorption by alga I was estimated after time-intervals varying from 2 to 200 hours, the concentration, &c., being kept constant. After 24 hours a steady state is established, but it is not known whether this is also the case in the runs with the other salt solutions or with the other algae. The adsorption of silver ions by alga I was measured both in an unstirred and a stirred suspension, the rate of adsorption being larger in the latter case.

In order to measure the metal ion adsorption by acid-extracted algal material under continuous flow conditions, the adsorbents equivalent to a dry weight of 25 g. were introduced into vertically supported glass tubes of 2.5 cm. diameter, and kept in position by a layer of glass wool and by a perforated rubber stopper. The solution, containing the ion to be adsorbed (feed solution), passed downwards and the adsorbent was kept completely immersed with the help of a constant-level device. The sedimentation volume of the fully swollen algal material, of the particle size specified on page 79, was 7–9 ml./g. If nothing else is mentioned, all experiments were done with 0.01 N feed solutions, the rate of flow being 800–1,000 ml./hour. Qualitative adsorption tests with the following algae were made: *Laminaria digitata* (frond and stipe), *L. saccharina*, *L. Cloustoni*, *Chorda filum*, *Fucus vesiculosus*, *F. platycarpus*, and *Ascophyllum nodosum*; and with the following solutes: ferric ammonium sulphate, ferric chloride, aluminium sulphate, chromic acetate, lead acetate, bismuth nitrate, manganese sulphate, copper sulphate, silver nitrate, calcium sulphate (and other calcium salts mentioned in Table III), magnesium chloride, sodium chloride, potassium chloride, barium hydroxide, ammonium hydroxide, and aniline hydrochloride. In all cases a marked cation adsorption could be detected and it was also established that the spent algae could be regenerated by rinsing with 1 N mineral acids. A few experiments with solutions containing both sodium and potassium ions were made, but no selective adsorption of potassium could be detected.

Systematic quantitative experiments were done with *Ascophyllum* (same batch as that used for the analyses of Table I) and with feed solution containing calcium ions. Measured portions of the effluent were collected after known time-intervals and in each portion acidmetric titrations and calcium determinations were made. It was thus possible to compute hydrogen-ion release values, β , break-through capacity, γ , and half-saturation values, δ , the latter quantities being defined by:

γ = number of equivalents of calcium ion adsorbed, until the calcium ion could just be detected in the effluent.

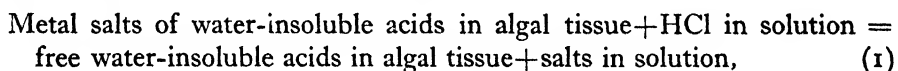
δ = number of equivalents of calcium ion adsorbed, until the calcium ion concentration of the effluent is one-half of that of the feed solution.

The dependence of the adsorption capacity on the particle size of the algal material and on the rate of flow, concentration, and acidity of the feed solution is shown by the figures in Table III.

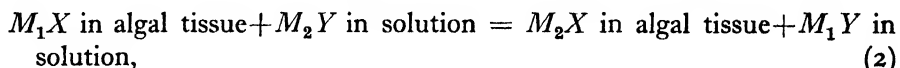
The γ and δ values depend also on the dimension of the adsorption bed (particularly its depth), and on the previous treatment of the algal material. The adsorbent used for the tests in Table III was stored for 3 weeks at room temperature; it was then extracted with 1 N hydrochloric acid, washed with water and placed for 2 weeks in 0.01 N calcium chloride. Before commencing the adsorption tests the acid treatment and the water wash were repeated.

DISCUSSION

The figures in the sixth, twelfth, and tenth lines of Table I show that on treatment of 1 kg. water-washed *Ascophyllum* with an excess of 1 N hydrochloric acid 2.5 ± 0.4 g.-equivalent metals dissolve, while 3.0 ± 0.3 g.-equivalent hydrogen ions are adsorbed, the alga containing 2.8 ± 0.2 g.-equivalent water-insoluble acids. Similar stoichiometric relationships characterize the reaction between hydrochloric acid and the other algae listed on page 80; all these observations can be represented by the equation:



where the dissolution of each equivalent of metal ions involves the adsorption of one equivalent of hydrogen ions. As regards the adsorption by the algal material which had not been in contact with hydrochloric acid, the figures in the sixth, seventh, and eleventh lines of Table II indicate that in these reactions the pH of the solution is not substantially decreased and that the metal ion release values, α , are near to unity. Simple stoichiometric relationships of this kind could hardly be expected if, under the present experimental conditions, the cation adsorption depended mainly on a slow diffusion through a membrane, but they can be explained by exchange processes of a type:



where M_1 and M_2 are metal ions in the exchange position, X is the anion of the water-insoluble acids (a) and (b), while Y are other anions.¹

Bird and Haas (1931) believed that the alginic acid in a species of *Laminaria* occurred in the free state, while Dillon and McGuinness (1931) and Miwa (1940) suggested salt formation. The experimental evidence on which these and other earlier contradictory conclusions are based is by no means satisfactory, however, and the results of the present adsorption tests, together with the swelling experiments (Wassermann, loc. cit.), appear to prove for the first time that these species do not contain free alginic acid. For if free alginic acid were present, reaction (2) would be accompanied by another process of

¹ If M_1X is not completely converted, mixed metal alginates of the type described in another note will be formed.

TABLE III

Calcium Ion Adsorption under Continuous Flow Conditions by Acid-extracted Ascophyllum; Influence of Particle Size of Alga, Rate of Flow, Concentration, and Acidity of Feed Solution

Ref. No.	Particle size of dry alga (mesh).	Rate of flow ml./hr.	Solute in feed solution.		Conc. in g.-equiv./l.		pH		β .	α	δ		
			a.	b.	a.	b.	Feed solution.	Effluent.†					
1	< 7* 7-25 25-52	800- 1,000 300 1,900	CaCl ₂	—	0.0100	6-6.5	2-2.2	1.05	< 0.1	0.5			
2								1.05	0.4	1.0			
3								0.90	1.0	1.7			
4	7-25				CaCl ₂		—	0.0038 0.011 0.028 0.012 0.010 0.010 0.011 0.010	0.010 — — 0.016 — — — —	1 6 6 6.3 12	0.99	0.5	1.1
5											0.99	0.3	0.9
6											1.0	0.8	1.2
7											1.05	0.5	1.2
8											—	0.3	1.2
9											—	< 0.1	< 0.1
10											1.05	0.5	1.0
11											1.0	1.3	1.6
12											—	1.3	1.7
13	—				2.6		3.4						

* Pieces of about 0.5 cm. length were used.

† The pH values in this column relate to the stage in which the calcium ion concentration in the effluent was still smaller than one-half of that in the feed solution.

the type of the reverse reaction (1); this, however, is incompatible with the results of the pH measurements listed in columns 1, 3, 5, &c., of Table II. It is suggested, therefore, that a complete neutralization of the various acids in the cell tissue has taken place, with the participation of some or all of the principal metals listed in Table I.

On extraction of the algae with 1 N hydrochloric acid the acidic groupings of the alginic acid and of the other water-insoluble acids are set free, and, therefore, a metal ion adsorption by hydrochloric acid extracted algae is always accompanied by a release of hydrogen ions to the solution (see the pH values listed in the relevant columns of Table II). Under static conditions, a hydrogen ion release value of 1 was obtained in one experiment only, the smaller ratios observed in the other runs being perhaps due to a slow consecutive reaction of the released acid. In a continuous flow secondary changes are less likely to affect the over-all results of the adsorption measurements, and it is understandable, therefore, that the hydrogen release values, β , as determined under these latter conditions, are near to unity (see Table III).

These results make it probable that reaction (1) is reversible and that the metal ion adsorption by hydrochloric acid extracted algae is due to a partial neutralization of the free water-insoluble acids in the cell tissues.

One of the algae here considered contains about 2.8 g.-equivalent of such acids/kg. dry weight (see Table I), while the figures in the ninth line of Table II vary in most cases between 0.2 and 0.5, the minimum and maximum values being 0.04 and 1.6 g.-equivalents. It is probable, therefore, that neither the reverse reaction (1) nor reaction (2) has gone to completion and that under these conditions the potential adsorption capacity of the algae has not been reached. It is also of relevance in this connexion to note that most of the break-through capacities, γ , and half-saturation values, δ , listed in Table III, vary between 0.3 and 1.2 g.-equivalent, the calcium appearing in the effluent before the 'neutralization' of the adsorbent is complete. In run No. 13 of Table III, the pH of the solution was relatively high; under such conditions the break-through capacity increases to a value approaching the figure in the tenth line of Table I,¹ the adsorption capacity of the algae thus becoming similar to that of certain recognized base exchange materials.

SUMMARY

Adsorption experiments are described which show that alginic acid (*a*) and other water-insoluble acids (*b*) occur in the cell tissue of brown algae in the form of various metal salts and not in the free state. On treating the algae with 1 N hydrochloric acid the metals dissolve, while the hydrogen ions, necessary for setting free the acidic groupings of (*a*) and (*b*), are taken up from the solution. If the hydrochloric acid extracted and subsequently water-washed algae are brought into contact with metal salt solutions, the reverse process takes place, namely, an adsorption of metallic cations and a release

¹ The half-saturation value is even larger, which is possibly due to salt formation with acids liberated as a result of alkaline ester hydrolysis.

of hydrogen ions. This type of ionic exchange adsorption has been studied in a static system and, more thoroughly, in a continuous flow. Under the latter conditions the adsorption capacity depends *inter alia* on the particle size, pre-treatment, concentration, acidity, and rate of flow.

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Studies in the Physiology of Obligate Parasitism

I. The Stimuli determining the Direction of Growth of the Germ-tubes of Rust and Mildew Spores

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With one Diagram in the Text

INTRODUCTION

THE obligate parasitism of the Rust, Powdery, and Downy Mildew Fungi is of an intimate nature, and a knowledge of the physiological relationships between host and parasite is needed. Some observations on physiological aspects of the host-parasite complex have been recorded, but a handicap has been the difficulty of studying the fungus apart from the host plant. Some workers have related the presence of haustoria with the obligate nature of the parasitism, while others have postulated a specialized type of nutrition in these fungi. The experiments described in this series of papers should lead, it is thought, to some knowledge of the physiology of these parasites, and hence to an understanding of the host-parasite relationship.

Following Balls' (1905) demonstration, by the use of punctured rubber membranes, of positive hydrotropism in the germ-tubes of the uredospores of *Puccinia glumarum*, this tropism has been considered the chief factor in directing the growth of the germ-tubes towards the stomata of the leaf. The reaction described by Balls was growth along an increasing relative humidity gradient. Balls did not suggest that this necessarily meant growth into a water surface. Robinson (1914), studying the sporidia of *P. malvacearum* immersed in drops of water, found that their germ-tubes grew out of such drops, and made a tentative suggestion that negative hydrotropism and negative heliotropism were acting. He also considered the possibility of this direction of growth being due to a gradient of oxygen or of carbon dioxide, such as Corner (1935) has suggested for *Erysiphe graminis*. Corner in some of his experiments with this fungus observed the germ-tubes standing erect like a forest of telegraph poles. Johnson (1934) described the growth of germ-tubes of *P. graminis* when in contact with the leaf surface as at right angles to the longer axis of the epidermal cells. He attributed this directed growth to a thigmotropic stimulus. There appear to have been no observations on the direction of growth of the germ-tubes of the downy mildews.

EXPERIMENTS USING PETRI DISHES

General experience has shown that the direction of the early growth of the germ-tubes of most fungal spores, if germinating on the surface of a liquid

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or a solid medium, is along the surface or into the medium; aerial hyphae are produced later. Corner (1935) germinated mildew spores on water in dishes and described the upright growth of the germ-tubes, i.e. growth away from the surface, as abnormal for the fungi. A slight tap on the edge of the dish, he states, was sufficient to cause the germ-tubes to fall over, and so appear to have grown along the surface. This growth away from the surface I have found to occur under certain conditions in all species of powdery mildews tested. A similar behaviour was noticed when the aecidiospores and uredospores of a number of rust fungi were germinated.

As an example, an experiment with *E. graminis* will be described (see Table I). A thin layer of 2 per cent. gelatin was poured into a Petri dish

TABLE I

Direction of Growth of the Germ-tubes from Spores of Erysiphe graminis germinated Overnight on the Surface of 2 per cent. Gelatin in Glass Rings in Petri Dishes

Rings.	No. of spores germinated.	No. of germ-tubes growing upwards.	No. lying along the medium.	Doubtful.
1	57 (101)	48	7	2
2	184 (281)	153	25	6
3	200 (283)	172	12	16
4	68 (109)	51	8	9
5	79 (117)	61	12	6
6*	85 (127)	63	19	4
7	67 (102)	55	6	6
8	65 (114)	60	1	4
9	60 (108)	49	8	3
Totals	865 (1342)	712	98	56
%	64	82	11	7

The numbers in parentheses show the total number of spores employed.

* The difference between the spore and germ-tube numbers is probably an error of counting.

with a close-fitting lid. A number of glass rings, $\frac{1}{2}$ in. in depth, were put into the dish, and the lid replaced. When the gelatin had set, the dish was placed in a humidity chamber (R.H. c. 98 per cent.) and the lid removed. Fresh mildew spores gathered on a camel-hair brush were then allowed to fall on the gelatin within the glass rings. The lid was at once replaced and the dish left overnight on the stage of a microscope. Next morning, without shaking the dish, the number of germinated spores and the number of germ-tubes which had grown away from the gelatin surface were counted. With a germination of 64 per cent., some 82 per cent. of the germ-tubes had so grown. Those whose position was uncertain were probably those which had fallen over owing to vibration.

To ascertain how common such behaviour was in rust, mildew, and other fungi, a range of genera and species was tested. The result of this survey is shown in Table II. It appeared that this phenomenon is general in both rusts

and powdery mildews, but is perhaps confined to them as the other species of fungi tested did not show it.

TABLE II

Direction of Growth of Germ-tubes of a number of Rust, Mildew, and other Fungi when growing in Petri Dishes on 2 per cent. Gelatin. Counts after 8-12 Hours

Fungus.	Type of spore.	Percentage germination.	Percentage of germ-tubes growing away from medium.
<i>Melampsora Euonymi-Caprearum</i> Kleb.	aecidiospores	89	81
<i>M. Rostrupii</i> Wagner.	aecidiospores	49	95
<i>Coleosporium Sonchi</i> Lev.	uredospores	92	75
<i>Phragmidium discolorum</i> James.	aecidiospores	82	90
<i>Puccinia Pruni-spinosae</i> Pers.	aecidiospores	80	100
<i>P. violae</i> (Schum.) DC.	aecidiospores	79	100
<i>P. glumarum</i> (Schum.) Erikss and Henn.	uredospores	87	100
<i>P. tritici</i> Erikss.	uredospores	92	100
<i>P. graminis</i> Pers.	uredospores	92	100
<i>Uromyces Poae</i> Raben.	aecidiospores	60	90
<i>U. Dactylidis</i> Oth.	aecidiospores	26	86
<i>Podosphaera leucotricha</i> (Ell. et Er.) Salm.	conidia	40	84
<i>P. Oxyacanthae</i> (DC.) de Bary.	conidia	48	100
<i>Sphaerotheca mors-uvae</i> (Schw.) Berk.	conidia	42	100
<i>S. pannosa</i> (Wallr.) Lev.	conidia	70	100
<i>Erysiphe graminis</i> DC.	conidia	99	100
<i>Microsphaera Alni extensa</i> Salm.	conidia	95	100
<i>Peronospora Schachtii</i> Fuckel.	conidia	93	0
<i>P. parasitica</i> (Fr.) Tul. (Pers. ex Fr. Tul.)	conidia	81	0
<i>Bremia lactucae</i> Regel.	conidia	76	0
<i>Aspergillus niger</i> van Tiegh.	conidia	89	0
<i>Penicillium expansum</i> Thom.	conidia	91	0
<i>Botrytis cinerea</i> Fr. (Pers. ex Fr.)	conidia	89	0
<i>Ustilago avenae</i> (Pers.) Jens.	spores	77	0
<i>Cladosporium fulvum</i> Cooke	conidia	67	0

Similar results were obtained with distilled water, tap-water, 2 per cent. sucrose, 1.5 per cent. agar, 15 per cent. gelatin, various stock media, and the leaves of a number of plants (not necessarily the proper host plant of the fungus), both *in situ* and in dishes. It was difficult to demonstrate growth away from the leaf surfaces *in situ*, and impossible at times to prevent the deposition of water drops on such surfaces. Certain precautions were necessary for a successful demonstration. The dish had not to be shaken, it had to be kept closed with a well-fitting lid, and, particularly in the case of rusts, observations had to be made before the germ-tubes had grown too long. Otherwise, rust germ-tubes (depending to some extent on the species) formed a tangled mass of aerial hyphae, the individual hyphae of which could with

difficulty be traced as having grown away from the medium at first but later had shown 'looping', i.e. a falling over until they rested on other hyphae or upon the medium.

To determine the effect of gravity on this early direction of growth similar experiments were made with inverted dishes. There was no significant difference in the amount of growth away from the surface between the normal and inverted dishes, so gravity apparently had no influence. The effect of light was tested by putting dishes into an unlighted incubator at room temperature. No differences could be detected. From a consideration of the variety of media used it seemed improbable that the direction of growth could be ascribed to negative chemotropism. Another possible factor controlling the direction of growth was a gradient of oxygen or of carbon dioxide (Corner, 1935; Robinson, 1914). This factor was difficult to control experimentally. Examination of the direction of growth from isolated spores and from spores in groups showed that the previous observations were true for germ-tubes from isolated spores. However, the growth of germ-tubes from spores in groups was frequently away from the group, while under the same conditions (see later experiments) germ-tubes from isolated spores grew along the surface. Such a direction of growth might well be due to a gradient of oxygen or carbon dioxide. A distinction has therefore been made between isolated and grouped spores. All subsequent observations recorded in this paper refer to the direction of growth of germ-tubes from isolated spores, that is, spores separated from other spores by a distance greater than their longer axis. It was thought unlikely that any appreciable carbon dioxide gradient would be produced from an isolated spore. Finally as hydrotropism (Balls, 1905; Robinson, 1914) and thigmotropism (Johnson, 1934) have been observed in these fungi, it is possible that the direction of growth of the germ-tubes away from the surface of a medium may be due to one or more of these tropisms.

EXPERIMENTS USING MEMBRANES

Modified Van Tieghem cells (see Diagram 1) were used to investigate the effects of humidity conditions, &c., on the direction of growth of the germ-tubes. These air-tight cells differed from the usual type in having horizontal partitions made from thin collodion or gelatin membranes. The rings composing them were fastened together with vaseline, the membrane being mounted on the lower ring. Glass, vulcanite, and metal rings have been tried. As collodion membranes split more frequently when mounted on glass than on metal rings, the latter have been mainly used. Two drops of collodion solution (0.2 per cent. in ethyl alcohol/ether 30/70 mixture) were used to make the membranes (with a diameter before mounting of 2.5 cm.) by placing them on a clean, level, dry glass sheet. Some of the solvent was then allowed to evaporate, the amount being controlled by the duration of this drying process. Afterwards the glass sheet was immersed in water. In doing this, the edge of a film of water was allowed to run between the membrane and the glass, so floating the membrane off the glass. The membranes were lifted from the

water surface on the rings and the superfluous water drained away. Wetting the membrane surface did not alter its characteristics, but it was difficult to get rid of the water drops on the membrane surface without reducing the water content of the membrane. The membrane carried a drop of distilled water on its under side, while the spores were placed on its upper surface over the water drop. In some experiments a ring of filter-paper was made to adhere to the coverslip lid of the upper chamber by being moistened with a drop of water or with sulphuric acid, control of the humidity gradient being

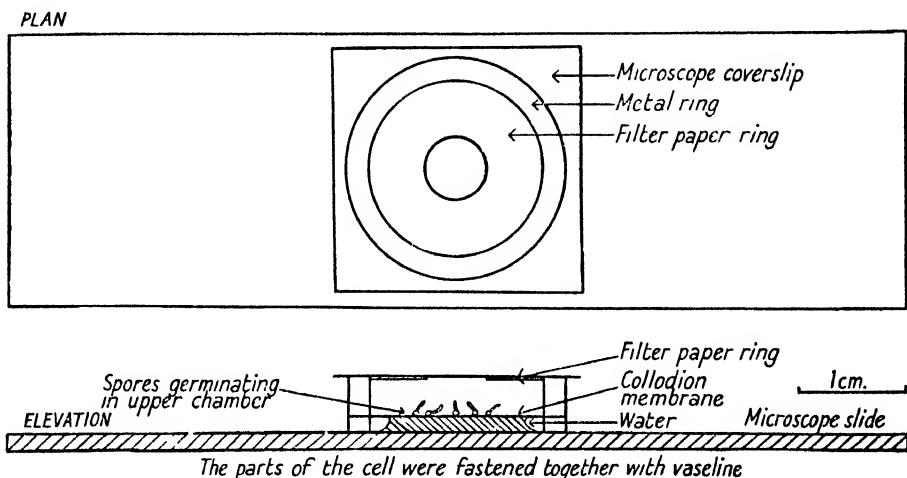


DIAGRAM I. Van Tieghem drop cell, modified to provide a double-chambered air-tight cell.

obtained by varying the acid concentration. Observations on the direction of growth were made through the central hole in the filter-paper ring, after 8-12, 16-24, and 34-48 hours.

Consistent results were obtained when the modified Van Tieghem cells were set up in a humidity chamber (R.H. *c.* 98 per cent.) at room temperature in a dull light. This also rendered unlikely the existence of any appreciable humidity gradient at the start of an experiment. Under such conditions it was unnecessary to use wet filter-paper rings on the coverslips, but this precaution was taken at the start of all new experiments. The time taken to reach an equilibrium was not measured, but as the volume of the upper chamber was 1.3 c.c. and the evaporating surface was 3.1 sq. cm., it was unlikely to be more than half an hour. These arrangements ensured a rapid adjustment to small changes in room temperature, and condensation was negligible even at the highest humidities. A measurement of the relative humidity in the upper chamber of these cells would be invaluable, but this has hitherto proved impossible as their depth was only 3 mm. The effect of convection currents seemed to be unimportant as consistent results have been obtained over a long period.

It has been mentioned that the degree of drying of the membranes is important. During this drying process membranes pass from a liquid to a solid state, and this change of state can be recognized by a difference in the reflection of light from the membrane surface. At a later stage of drying they turn first red-green, then bronze-blue. Membranes floated off the glass surface just before colouring developed were called 'pre-coloured' and those after the bronze-blue colour had appeared were called 'post-coloured'. The temperature was also important, and to obtain as uniform membranes as possible the process was always carried out at a room temperature of *c.* 17° C.

An experiment was made with *E. graminis* spores on pre-coloured membranes, to determine the consistency of the results for the direction of growth of the germ-tubes, those growing away from the membrane being counted (see Table III). Counts of eleven membranes gave a standard error of 5.4. Therefore it was decided that counts from six different membranes would be sufficient. This was repeated with membranes dried for a longer time, and the results (Table III) showed the importance of standardizing the degree of drying. It was difficult to determine accurately the appearance of the red-

TABLE III

Direction of Growth of the Germ-tubes of Isolated Spores of Erysiphe graminis when germinated on Membranes made from Two Drops of 0.2 per cent. Collodion in a 30/70 Alcohol/Ether Mixture, which were dried for Varying Times. The Temperature of Drying of the Membranes was 17° C. The Room and Humidity Chamber Temperature was 17° C. The Percentage Germination was 77. The Starting Relative Humidity in the Cells was 98 per cent.

Membranes floated off when:						
Pre-coloured. Drying time <i>c.</i> 30 secs.		Red-green. Drying time <i>c.</i> 45 secs.		Post-coloured. Drying time <i>c.</i> 60-90 secs.		
No. of germ- tubes counted on each membrane.	% germ- tubes growing away from membrane.	No. of germ- tubes counted on each membrane.	% germ- tubes growing away from membrane.	No. of germ- tubes counted on each membrane.	% germ- tubes growing away from membrane.	
74	66	87	23	112	18	
102	70	60	61	79	12	
99	79	54	48	94	11	
122	71	108	78	105	13	
153	71	121	59	99	8	
125	70	79	45	105	22	
104	61	—	—	—	—	
141	79	—	—	—	—	
109	66	—	—	—	—	
167	74	—	—	—	—	
107	69	—	—	—	—	
Summary	1,306	71	509	52	594	14
σ/\sqrt{n}	—	5.4	—	18.5	—	5.1

green condition and it is suggested that this was responsible for the higher variation obtained with such membranes.

It should be pointed out that the growth of the individual germ-tube was either along the membrane or away from it. The angle between the membrane and the 'upright' germ-tube, i.e. that growing away from the membrane, was not always 90° but seldom less than 60° , as estimated by eye through the microscope. Therefore the direction of growth must have been determined by a stimulus or stimuli since in the absence of stimuli, growth would be in all directions. As growth was in two directions, i.e. along the membrane surface or at an angle greater than 60° , it seemed probable that more than one stimulus was operative.

A comparison was now made of the size of the effect due to variation in the degree of drying of the membranes and that due to a humidity gradient. It was desirable to produce this humidity gradient without the use of acid on the cover-slip. This was done either by arranging for a continuous alteration in temperature, or by lowering the starting humidity, or by increasing the volume of the upper chamber of the cells. All methods meant an alteration in the actual rate of evaporation but no change in the condition of the membrane.

A rising temperature during the early part of an experiment was obtained by setting up the cells in the humidity chamber as usual, and transferring them to a refrigerator, the cooling mechanism of which was then turned off. The refrigerator took 8 hours to reach room temperature. It will be seen from Tables IV (*E. graminis*) and V (*P. triticina*) that the effect of the rise in temperature on the percentage of upright germ-tubes was slight as compared with that due to variation in the degree of drying. It may well be that the

TABLE IV

Direction of Growth of the Germ-tubes from Isolated Spores of Erysiphe graminis when germinated on Membranes. The Actual Rate of Evaporation was varied by a Rising Temperature for the First 8 Hours of Germination. The Percentage Germination was 82. Other details as in Table III

		Temperature of germination.									Summary. σ/\sqrt{n} .	
Membranes pre-coloured. Drying time <i>c.</i> 30 secs.	19° C.	{	No. germ-tubes									
	observed		115	112	107	112	87	82	615	—		
	From 6° to 19° C. in first 8 hours	{	% upright growth		67	73	73	65	67	82	69	2.5
			No. germ-tubes									
Membranes post-coloured. Drying time <i>c.</i> 60-90 secs.	19° C.	{	observed		142	174	198	106	128	104	852	—
	% upright growth		57	53	58	56	58	49	55	1.4		
	From 6° to 19° C. in first 8 hours	{	No. germ-tubes									
			observed		118	132	186	149	115	153	853	—
	19° C.	{	% upright growth		13	12	13	15	15	14	13	0.6
	No. germ-tubes											
	From 6° to 19° C. in first 8 hours	{	observed		140	154	89	124	125	131	763	—
			% upright growth		17	11	16	16	10	14	14	1.1

TABLE V

Direction of Growth of the Germ-tubes from Isolated Spores of Puccinia triticina germinating on Membranes. The Membranes were floated off 'post-coloured'. The Actual Rate of Evaporation was varied by a Rising Temperature for the First 7½ Hours of Germination. The Percentage of Germination was 94. Other details as in Table III

Temperature of germination.							Summary. σ/\sqrt{n} .
14° C.	No. germ-tubes observed	110	242	135	186	192	865 —
	% upright growth	70	74	73	76	73	73 1.0
From 5° to 14° C. in 7½ hours	No. germ-tubes observed	96	166	231	221	267	981 —
	% upright growth	61	61	66	58	59	61 1.3

humidity gradient was not altered by this slowly rising temperature. The experiment with a falling temperature failed as the spores did not germinate.

In Table VI are included the results obtained with rust, powdery, and downy mildews on pre-coloured and post-coloured membranes, when both the starting humidity and the volume of the upper chamber of the cell were altered. The effect was not so great when the starting humidity was altered from 98 to 30 per cent. R.H. as when the volume of the cell was changed from 1.8 to 28 c.c. Alterations of starting humidity and of cell volume did not effect the percentage of upright germ-tubes as much as did the change in degree of drying from pre-coloured to post-coloured (compare Table III).

In these experiments where humidity gradients were to be expected, angles of less than 60° appeared to be more common than in those experiments in which precautions were taken to prevent humidity gradients.

Having demonstrated that consistent results could be obtained with this method, and that the condition of the membrane—as determined by the degree of drying—had a major effect on the direction of germ-tube growth, the range of membranes used (see Table VII) was extended by including some gelatin media and membranes; the methods of making the latter will be described in a later paper. In addition the behaviour of the germ-tubes of certain other fungi was tested; these being selected mainly on account of their spores being airborne, so that they could be dropped and not brushed on to the membranes.

No germ-tubes of *Ustilago violacea* or *Aspergillus niger* were seen to grow away from any of these membranes, and the counts for *Monilia fructigena* and *Botrytis cinerea* did not reach 10 per cent. But the most striking feature was again the high percentage of upright growth, 100 per cent. in *E. graminis* and *P. triticina*, on 2 per cent. gelatin medium (see also Table II) and on water. There was some correspondence on collodion membranes between the degree of drying and the percentage of upright growth; the shorter the time

TABLE VI

Direction of Growth of the Germ-tubes from Isolated Spores of Erysiphe graminis, Peronospora Schachtii, and Puccinia triticina on Membranes made at 17° C. from Two Drops of 0.2 per cent. 30/70 Alcohol/Ether Collodion Solution, dried for the Former Two Species to 'Pre-coloured' (drying time c. 30 secs.) and for the Latter Species to 'Post-coloured' (drying time c. 60–90 secs.). The Actual Rates of Evaporation were altered by varying the Volume of the Enclosed Chamber and the Starting Relative Humidity. The Room and Humidity Chamber Temperature was 17° C. The Number of Membranes used in each case was 6. Water was placed on the Cover-slips

Volume of chamber 1.8 c.c.

		Starting R.H. 98%.	Starting R.H. 30%.
<i>E. graminis</i>	No. germ-tubes observed	934	897
	Upright growth	70	61
	σ/\sqrt{n}	1.2	2.9
<i>P. Schachtii</i>	No. germ-tubes observed	672	442
	Upright growth	62	39
	σ/\sqrt{n}	2.6	2.0
<i>P. triticina</i>	No. germ-tubes observed	392	322
	Upright growth	83	73
	σ/\sqrt{n}	0.8	3.1

Volume of chamber 28 c.c.

		Starting R.H. 98%.	Starting R.H. 30%.
<i>E. graminis</i>	No. germ-tubes observed	646	1,012
	Upright growth	47	28
	σ/\sqrt{n}	1.5	1.8
<i>P. Schachtii</i>	No. germ-tubes observed	329	467
	Upright growth	53	31
	σ/\sqrt{n}	1.7	2.4
<i>P. triticina</i>	No. germ-tubes observed	612	315
	Upright growth	53	22
	σ/\sqrt{n}	1.1	2.4

for drying, the higher in general the percentage of upright growth. On the gelatin membranes, those treated with 90 per cent. alcohol formalin showed a lower percentage of upright growth than the untreated, except for *Cladosporium fulvum*. An angle of over 60° between germ-tube and membrane, both collodion and gelatin, was seen in all these fungi. It was therefore clear that this phenomenon of upright growth was not confined to rust and powdery mildews, except on the 2 per cent. gelatin medium and water.

A close examination was then made of the spores of the various fungi on the 2 per cent. gelatin medium. It seemed possible that the spores of all the fungi used, except rust and powdery mildew, became wet relatively easily. It might then be that the germ-tubes of such fungi grew in the liquid and did not get out at once, and so the upright growth effect was masked. On a

TABLE VII

Direction of Growth of the Germ-tubes from Isolated Spores of Various Fungi on a Series of Membranes. In each case the Number of Germ-tubes observed was about 500, on Six Different Membranes

Membrane 0.2% 30/70 collodion.	Percentage of upright growth.									
	<i>Erysiphe graminis. triticea.</i>	<i>Puccinia triticea.</i>	<i>Peronospora Schachtii.</i>	<i>Peronospora parasticta.</i>	<i>Cladosporium fulvum.</i>	<i>Verticillium Dahliae.</i>	<i>Ustilago violacea.</i>	<i>Aspergillus niger.</i>	<i>Botrytis cinerea.</i>	<i>Monilia fructigena.</i>
Pre-coloured .	68	95	62	36	90	46	0	0	9	4
Post-coloured .	14	73	26	32	51	40	0	0	5	2
Dried on glass for 10 minutes .	13	12	14	35	37	7	0	0	7	2
Post-coloured, and then dried for 24 hours .	30	68	11	4	12	2	0	0	3	0
Plain gelatin mem- brane .	35	95	89	33	10	35	0	0	5	0
Soaked in 90% al- cohol formalin for 24 hours .	32	71	12	3	12	5	0	0	0	0
2% gelatin medium	100	100	0	0	0	0	0	0	0	0
Water .	100	100	0	0	0	0	0	0	0	0
Germination per- centage .	74	86	90	76	65	64	92	81	84	63

gelatin or a collodion membrane part of the surface is water and part solid, consequently the germ-tubes would be likely to come into contact with a solid or a water surface without the spore becoming wet. This suggestion has been tested (Table VIII) by germinating the spores of four of the fungi on pre-coloured membranes with a film of water on the upper surface. Only in *E. graminis* did any appreciable growth away from the membrane take place. It was therefore concluded that the exceptional behaviour of rust and powdery mildew on 2 per cent. gelatin medium and water was due to the relative difficulty of wetting their spores.

TABLE VIII

Behaviour of Germ-tubes of Isolated Spores of Various Fungi, germinated on Pre-coloured Collodion Membranes. The Starting Relative Humidity was 98 per cent. All were examined after 18 Hours. The Number of Germ-tubes counted was approximately 500, in each case on Six Different Membranes

Membrane.		<i>Erysiphe graminis.</i>	<i>Peronospora Schachtii.</i>	<i>Botrytis cinerea.</i>	<i>Cladosporium fulvum.</i>
Pre-coloured, dry at the start	Percentage upright growth	71	65	10	84
Pre-coloured, wet at the start	Percentage upright growth	65	4	0	0

The conclusions so far reached were that with constant humidity conditions the percentage of upright growth was determined by the condition of the collodion or gelatin membranes. Now it is well known that the concentration of alcohol in which collodion is soaked will affect the size of the pores, and the effect of such treatment of the membranes was tested. They were soaked for 12 hours in a range of alcohol concentrations from 95 per cent. downwards, and afterwards washed for 24 hours in distilled water. Care was always taken to see that the membrane surface was dry before starting an experiment; 0.4 per cent. collodion was used because 0.2 per cent. membranes tended to break up in the higher alcohol solutions.

In addition to testing the effect of soaking the membrane in alcohol, drops of sulphuric acid of varying strengths were placed on filter-paper rings on the coverslips to provide a series of humidity gradients. The cells were replicated four times, and in view of their numbers it was decided to rely upon observation rather than on counts of the percentage of upright growth in the germ-tubes. All the cells in each experiment were made and kept in a humidity chamber (R.H. *c.* 98 per cent.) except while under observation.

The results (Tables IX and X) showed that the direction of growth of the germ-tubes was away from the surface of those membranes soaked in 95 per cent. alcohol, when the liquid used on the cover-slip was water or dilute acid. As the alcohol concentration was lowered, so the direction of growth

TABLE IX

Direction of Growth, 8–12 Hours after Germination, of the Germ-tubes of Isolated Spores of Erysiphe graminis when germinated on Membranes made from a 0.4 per cent. Collodion Solution in a 9/91 Alcohol/Ether Mixture. These Membranes were soaked for 12 Hours before use in Varying Strengths of Alcohol. In all cases when the Direction of Growth was both away and along the Membrane, 'Loops' were observed. The Surface of all Membranes was dry at the Start of the Experiment

Percentage alcohol in which the membranes were soaked before use.	Liquid on cover-slip.			
	1 drop of H ₂ O.	1 drop of 1% H ₂ SO ₄ .	1 drop of 2.25% H ₂ SO ₄ .	1 drop of 5% H ₂ SO ₄ .
95	All away from membrane	Some away, some along membrane	Some away, some along membrane	All along membrane
90	All away from membrane	Some away, some along membrane	All along membrane	All along membrane
80	Some away, some along membrane	All along membrane	All along membrane	All along membrane, some inhibition of germination
60	Some away, some along membrane	All along membrane	All along membrane	All along membrane, some inhibition of germination
40	Some away, some along membrane	All along membrane	All along membrane	All along membrane, some inhibition of germination
Water	All along membrane	All along membrane	All along membrane	All along membrane, some inhibition of germination

was more and more along the surface. Finally, with membranes soaked in water, even when water was used on the cover-slips, growth took place along the surface. Under intermediate conditions, when the direction of growth included germ-tubes growing away from as well as along the membrane surface, examples of 'looping', or growth of a single germ-tube at first away from and then towards the membrane, were frequently seen. A few cases of 'reverse looping', or growth along the membrane by a single germ-tube and then away from the membrane, were noticed, but these were insufficient to justify any deduction. As the concentration of acid was increased, so the tendency for growth along the membrane became more marked. This was the more noticeable as the alcohol concentration was decreased. No conclusions were reached as to the angle between the upright germ-tube and the membrane, except that it was almost invariably 90° on the high-alcohol-treatment membranes with water on the cover-slip. These experiments have

TABLE X

Direction of Growth, 6–8 Hours after Germination, of the Germ-tubes of Isolated Uredospores of Puccinia triticina when germinated on Membranes made from a 0.4 per cent. Collodion Solution in a 9/91 Alcohol/Ether Mixture. These Membranes were soaked for 12 Hours before use in Varying Strengths of Alcohol. In all cases when the Direction of Growth was both away and along the Membrane, 'Loops' were observed. The Surface of all Membranes was dry at the Start of the Experiment

Percentage alcohol in which the membranes were soaked before use.	Liquid on cover-slip.				
	1 drop of H ₂ O.	1 drop of 2.25% H ₂ SO ₄ .	1 drop of 5% H ₂ SO ₄ .	1 drop of 10% H ₂ SO ₄ .	1 drop of 22.5% H ₂ SO ₄ .
95	All away from membrane	All away from membrane	Some away, some along membrane	Some away, some along membrane	All along membrane
90	All away from membrane	All away from membrane	Some away, some along membrane	All along membrane	All along membrane
80	All away from membrane	Some away, some along membrane	Some away, some along membrane	All along membrane	All along membrane
60	Some away, some along membrane	Some away, some along membrane	All along membrane	All along membrane	All along membrane
40	Some away, some along membrane	All along membrane	All along membrane	All along membrane	All along membrane
Water	All along membrane	All along membrane	All along membrane	All along membrane	Some inhibition of germination

thus shown that a control of the direction of growth of the germ-tubes can be obtained either by altering the acid concentration on the cover-slip (i.e. the humidity gradient) or by varying the previous alcohol treatment of the membranes.

DISCUSSION

When precautions were taken to prevent humidity gradients, upright growth of the germ-tubes of rusts and powdery mildews was observed from the surface of water, 2 per cent. gelatin medium, pre-coloured, and high-alcohol-treated collodion membranes. The direction of growth was along the surface of post-coloured and low-alcohol-treated collodion membranes. In both cases the growth was clearly affected by a stimulus, for its direction was in no case haphazard except perhaps when there was a humidity gradient. The stimulus controlling this directed growth could not have been chemotropic in nature, because parallel results were obtained with both collodion and gelatin. It could not have been due to gravity or light, for the percentage

of upright growth was the same in inverted dishes and in dishes kept in an incubator. It could not have been due to a gradient of oxygen or carbon dioxide, as observations were limited to germ-tubes from isolated spores. The growth directed along the surface, in experiments where there were humidity gradients, could have been, and probably was, due to positive hydrotropism. In other experiments, where every precaution was taken to ensure that no humidity gradient was acting, this direction of growth along the membrane was due to some stimulus. The most probable stimulus would be a form of surface or contact stimulus. The direction of growth away from the surface of other membranes, when similar precautions had been taken, would again appear to be a response to contact, since the angle between germ-tube and membrane was usually over 60° . Thus the factor which affected the percentage of upright growth and the type of contact response was the condition of the membrane, as determined either by the duration of the drying process or by the concentration of the alcohol in which the membranes were treated.

The ordinary tropisms (geotropism, phototropism, &c.) are growth movements in response to a stimulus which has a direction of action. So one can properly speak of positive, negative, and dia-geotropism. A surface or contact stimulus has no direction of action, but the consequent growth movement has a direction either along or away from the surface responsible. A contact stimulus is called 'thigmotropic' in this paper, and the growth movement along a membrane caused by such a stimulus is called dia-thigmotropism, while the growth movement away from a membrane resulting from a contact stimulus is called negative thigmotropism.

The experiments with downy mildews and other fungi were not as numerous as those with rust and powdery mildew. However, the behaviour of *Peronospora Schachtii*, *P. parasitica*, *C. fulvum*, and possibly *V. Dahliae* was similar to that of *P. tritici* and *E. graminis*, and in contrast to that of the other fungi. It is noteworthy that the condition of the gelatin membrane affected the percentage of upright growth of *P. Schachtii* more even than that of *P. tritici* and *E. graminis*. It would seem that the thigmotropic stimulus varies with the material as well as the condition of the membrane.

This thigmotropic phenomenon has been observed only in the germ-tubes, and it cannot be assumed that it occurs also in the mature hyphae. To demonstrate this without growing the fungi on artificial media is difficult. That it is possibly the case in rusts would appear from the observation that infected leaves placed in conditions of high humidity show occasional growth of the hyphae from out of the infection spot.

If, however, the mature hyphae do in fact behave like the germ-tubes, then the occurrence of this thigmotropic phenomenon must exercise a profound effect on the habit of growth and the parasitism of these fungi. This deduction was made before it had been found that the phenomenon occurred in fungi other than rusts, e.g. *Cladosporium fulvum*. Certainly it is to be expected that the cultivation of these fungi on artificial media could only be achieved

by altering the condition of the normal surface of such media; otherwise growth would be away from the surface and no absorption of foodstuffs could occur. Such an alteration of the condition of the surface can be made by interposing a suitable membrane between fungus and nutrient medium. Such a device simulates the system of cell-wall and cell-contents to be found in the host plant. When this was done other reactions took place, such as the penetration of the membrane, nuclear division, haustorial formation, &c. Later papers will deal with these observations. It may be added that either the nutritional or the growth-factor requirements of rusts and mildews are available in culture media as normally prepared.

The two types of thigmotropic response, depending on the nature of the membrane surface, also suggest that more attention should be paid to the cell-wall and its effects on the parasite than has been done hitherto. The cell-wall would appear to influence the spread of parasites in infected tissues not merely as a physical barrier but as controlling the direction of growth of the hyphae through contact stimulation.

The growth of the germ-tubes away from a water surface in the rusts and mildews may throw light on some of their peculiarities. Powdery mildews are more common in dry seasons than in wet seasons. It is suggested that in wet seasons, on the average, there will be a greater area of water film on the leaf surfaces than in dry seasons. Consequently the mildew hyphae will tend to grow away from rather than along and in contact with them. In rusts and downy mildews the mature hyphae grow over the surfaces of the mesophyll cells of an infected leaf. If the mature hyphae, like the germ-tubes, grow away from a wet surface it follows, if no other factors are operative, that the surface of the mesophyll cells bordering the intercellular space must not be wet. This deduction was reached on the above evidence, and a similar conclusion was reached by Lewis (1938), who demonstrated that the surface of the wall of the mesophyll cells, which lines the intercellular spaces, was covered by a non-wettable layer.

Lastly, it must be emphasized that as this thigmotropic phenomenon has been observed in other fungi it is unlikely to be solely responsible for the particular type of parasitism found in rusts and mildews.

SUMMARY

The germ-tubes of rusts and powdery mildews grow sometimes along and sometimes away from the surface on which the spores have germinated. They grow away from the surface of water, and away from or along the surface of collodion or gelatin membranes according to the adjustment of the humidity gradient. When precautions are taken to prevent humidity gradients, the direction of growth with collodion or gelatin membranes is either along or away from the surface according to the previous treatment of the membranes.

The direction of growth of the germ-tubes of downy mildews is similar to that of rusts and powdery mildews, except when germinated on water, when they grow beneath the surface. It is suggested that this difference is due to

the fact that the spores of downy mildews are more easily wetted than those of rusts and powdery mildews.

It is concluded that the tropisms controlling the direction of growth of the germ-tubes are positive hydrotropism and two types of growth response due to contact; these are termed negative thigmotropism (growth away from a surface) and dia-thigmotropism (growth along a surface).

It is suggested that for the artificial culture of these obligate parasites a suitable membrane should be interposed between the fungus and the nutrient medium, thus simulating to some extent the system of cell-wall and cell-contents of the host plant.

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The Effect of the Plant Growth-regulator 4-Chloro-2-methylphenoxyacetic Acid on Mitosis in the Onion (*Allium cepa*)

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With Plates I and II

INTRODUCTION

REDUCTION of plant growth by the sodium salt of 4-chloro-2-methylphenoxyacetic acid ('Methoxone', M.C.P.A.) was reported by Templeman and Sexton (1946). This substance was shown to be highly selective in its action, causing serious injury to some plants, e.g. charlock, when applied at very low rates, while others, e.g. oats, were only slightly affected by much higher rates. Laboratory examinations have shown that M.C.P.A. reduces the root growth of cress at 0.01 p.p.m. and of wheat at approximately 0.5 p.p.m., when seeds of these species are germinated and allowed to grow for 4 days on agar media containing the active substance. At higher concentrations M.C.P.A. causes swelling of the regions of the roots immediately behind the tips. Since such swellings are also commonly produced by various mitotic poisons it was considered that the inhibition of root growth produced by M.C.P.A. might be related to its action on mitosis. The present paper therefore represents the first stage in an investigation into the mode of action of M.C.P.A. from the cytological point of view, and deals with the cytological effects produced by M.C.P.A. in the root-tips of the onion (*Allium cepa*), a susceptible species. Since the treatment times used in the present investigation were less than those used in the agar tests, a wider range of concentrations of M.C.P.A. has been employed.

METHODS

The following methods were used:

Onion bulbs were grown over distilled water in 300 c.c. conical flasks until the roots attained a length of approximately 1 cm. They were then transferred (after removal of excess water by means of filter-paper) to 4 in. \times 1 in. flat-bottomed specimen tubes containing the experimental solutions prepared from the pure sodium salt of 4-chloro-2-methylphenoxyacetic acid; only the roots were actually immersed in the solutions. When desired the onions were, after treatment, re-transferred to distilled water in 300 c.c. conical

flasks, the roots being carefully washed with distilled water to remove any free experimental solution from the surface of the roots before transfer.

Roots were fixed, either immediately after treatment or after an interval of 24–72 hours, during which time they were kept in distilled water.

Slides were prepared from root-tips by the Feulgen technique according to the following schedule:

Root-tips were fixed for $\frac{3}{4}$ –1 hour in chromacetic acid (9 volumes of 1 per cent. chromic acid to 1 volume of 5 per cent. acetic acid). They were washed in frequent changes of warm water (35–40° C.) for at least 1 hour, transferred to cold N.HCl for a few minutes before hydrolysis in N.HCl at 60° C. for 18 minutes, then re-transferred to cold N.HCl for a few minutes and quickly washed in distilled water; stained for 1 hour in leucobasic fuchsin (prepared by a modification of Coleman's method), followed by at least 1 hour in SO₂ solution; squashed under the cover-slip in a drop of 45 per cent. acetic acid and warmed, after which slide and cover-slip were separated in 95 per cent. alcohol (in a 3 in. \times 1 $\frac{1}{4}$ in. tube or grooved jar) and thereafter dealt with separately.

Each was counter-stained in 1 per cent. light green or 0.6 per cent. fast green FCF (in 80 per cent. alcohol) for 1 sec. or 6 sec. respectively, taken through 2 changes each of 95 per cent. alcohol, absolute alcohol and xylene, and slide and cover-slip were then recombined with Canada balsam in xylene.

After 48 hours on a hot plate at 35–40° C. the slides were sufficiently hardened to permit of examination under oil.

Preparations were examined by means of a Leitz microscope, using for critical work a $\frac{1}{12}$ -in. oil immersion objective (N.A. 1.32) and a 10 \times compensating eyepiece, illumination being by means of a Leitz microscope lamp, the light being dimmed by contrasting orange (Wratten 77A) or green filters.

For photographic work the same microscope was used, with a $\frac{1}{8}$ -in. dry objective and 10 \times eyepiece but no camera lens; the camera extension was 11 in., giving a magnification of 480 \times . Photographs were taken on Ilford Process Panchromatic plates.

RESULTS

When root-tips of onion are treated with M.C.P.A. for 22 hours and fixed immediately after treatment no cytological effects are produced at concentrations below 100 p.p.m. At 100 p.p.m. the first signs of longitudinal contraction and transverse swelling of the chromosomes are seen, accompanied by delayed separation of the chromatids at metaphase, leading to pairs in which the members lie closely parallel. At the same time the chromosomes become 'sticky', i.e. they appear to lose their rigidity and their surfaces become adhesive, so that they show curvatures and fuse where they come into contact. In the interphase nuclei pointed projections are often seen on the nuclear surface giving a star-shaped appearance.

At 500 p.p.m., chromatin bridges ('sticky bridges') are formed at anaphase

between the separating groups of chromosomes and these often persist into telophase (Pl. I, Fig. 1). In either case, portions of the bridge may be separated as fragments and, not being incorporated in either daughter-nucleus, form micro-nuclei which may be included in either daughter-cell in addition to the normal nucleus (Pl. I, Fig. 2).

At 1,000 p.p.m. the picture is very similar to that seen at 500 p.p.m. except that 'stickiness' is very much more pronounced.

At 2,500 p.p.m. the effects of extreme 'stickiness' are apparent, the chromosomes having lost their identity and run together into large, deeply staining, pycnotic masses of irregular shape. Cell division has ceased, all nuclei being seen at interphase. Buds of intensely Feulgen-positive material are extruded from the interphase nuclei (Pl. I, Fig. 3), and, being separated in the cytoplasm, form Feulgen-positive masses of varying shapes and sizes. Finally the whole cytoplasm becomes feebly Feulgen-positive and contains a large number of Feulgen-positive granules (Pl. I, Fig. 4). At this stage the nuclei often stain feebly with leucobasic fuchsin.

There appears to be a progressive reduction in the rate of cell division with increasing concentration, the number of mitoses visible on slides from the treatments below 100 p.p.m. approaching that of controls; while at 2,500 p.p.m. cell division has ceased. As long as nuclear division continues, however, the groups of daughter chromosomes separate at anaphase, even after the onset of 'stickiness'.

With increasing concentration there is also a progressive enlargement of the nucleolus relative to nuclear size, the nucleolus being, after the highest treatment, of enormous size; while, at this dosage, the nucleus itself becomes enlarged in relation to cell size.

It should be pointed out that at the lower treatments the effects described above are not to be seen in all cells at a given treatment, but that maximum effects occur in a proportion of cells, while others show milder effects or even, in many cases, normal division.

The mitotic irregularities induced by M.C.P.A. appear to be similar in many respects to the effects produced by X-irradiation on mitosis. As Marquardt (1938) has shown that the X-irradiation effects may be classified into two types—physiological and structural changes—further experiments have been carried out to determine whether the analogy with X-rays could be substantiated, by using high concentrations of M.C.P.A. for short periods (e.g. 2,500 p.p.m. for $\frac{1}{2}$ –4 hours) and fixing immediately on completion of treatments and at 24–72 hours after the end of treatment. With these shorter periods of treatment the results are, however, rather more variable. This may be due to differences in rates of diffusion of M.C.P.A. into different root-tips or perhaps to the lower ratio of treatment time to mitotic period.

(a) Immediate fixation

These results are similar to those already described, except that after 4 hours, the longest period of treatment, some nuclei are still dividing. By

comparing these short-period treatment preparations with preparations from the 22-hour treatment, it appears that a given dosage (i.e. concentration \times time) produces approximately the same effect, whether administered as long treatment at a moderate concentration or a shorter period at a high concentration.

(b) *Fixation at various time-intervals after treatment*

When onion root-tips which have been treated with M.C.P.A. at 2,500 p.p.m. for short periods ($\frac{1}{2}$ –4 hours) are replaced in water for 24–72 hours before fixation, some of the nuclei show the effects already described above. In others there appears to be neither swelling nor 'stickiness' of the chromosomes, their staining is weak, and paired daughter-chromosomes (Pl. II, Fig. 5), closely resembling the 'c-pairs' described by Swedish cytologists, are of frequent occurrence, when anaphases are rare.

The most interesting effects of 'recovery' are, however, the occurrence of irregular chromosome configurations at metaphase and anaphase. These effects, which are evidently due to chromatid interchange (i.e. irregular reunion of sister or non-sister chromatids, following breakage), include the occurrence of acentric fragments (Pl. II, Figs. 6 and 8), dicentric chromosomes (Pl. II, Fig. 7) and chromatin bridges at anaphase. These bridges are completely free from 'stickiness'. Fragments, as usual, lag at anaphase and may form micro-nuclei.

Seventy-two hours after treatment, however, conditions have in many nuclei returned to normal, though in some, abnormalities are still to be seen (e.g. fragments, Pl. II, Fig. 6).

DISCUSSION

It is clear that mitotic irregularities are induced by M.C.P.A. The concentrations which produce these abnormalities are, however, relatively high when compared with data available for other susceptible species. In cress, for example, when seeds are germinated and allowed to grow for 4 days on an agar medium containing 0.01 p.p.m. M.C.P.A. significant reduction in root growth results. In the experiments described in the present paper, however, the exposure times were considerably shorter and this may in some degree account for the relatively high concentrations which are required to produce mitotic irregularities. A further point to be borne in mind is that although the onion is a susceptible species under field conditions it may perhaps be less susceptible than cress in the laboratory. Even taking account of these possibilities, further work is, however, still required to determine whether the inhibition of mitosis is causally related to the inhibition of root growth observed in the laboratory.

The mitotic aberrations which result from the action of M.C.P.A. appear to be closely similar to those reported in the literature as having been produced by X-rays. These include chromatid breaks and subsequent interchanges, the presence of fragments and micro-nuclei, the enlargement of the nucleolus, the apparent delay in the mitotic cycle at interphase or early prophase, and

the occurrence of 'sticky' chromosomes and 'sticky bridges' at anaphase (cf. Lea, 1946).

In regard to the action of X-rays, it has been concluded (Marquardt, 1938) that the effects produced are of two types—physiological effects, i.e. 'stickiness' of chromosomes, and structural changes, i.e. chromatid breakage. 'Physiological' effects are sometimes referred to as 'primary', and 'structural' as 'secondary'. According to Marquardt, 'the physiological effects but not the structural changes are exhibited by the cells already in division at the time of irradiation, and that the structural changes, but not the physiological effects, are exhibited by the cells which enter division after the expiration of the period of reduced mitotic activity which follows irradiation'. Although in the present study no quantitative data are available, a comparison of preparations fixed immediately on completion of treatment with those fixed 24 hours or more after treatment suggests that a similar picture may hold for M.C.P.A. action.

It is therefore of interest to inquire as to what is known of the differences between interphase or early prophase nuclei and those in other stages of division. In the light of present evidence these appear to be the presence of nucleolus and nuclear membrane, the extended (uncontracted) state of the chromosomes which bear the minimum amount of deoxyribonucleic acid, and the presence of sulphhydryl groups in the interphase or early prophase nuclei; at other stages of division the nuclear membrane and nucleolus have disappeared, chromosomes are contracted and bear their full amount of deoxyribonucleic acid, and sulphhydryl groups are at a minimum (Caspersson, 1946; Chalkley, 1937; Darlington, 1942; Davidson and Waymouth, 1944).

It is clear from the results described above that M.C.P.A. influences some of these changes, since the nucleolus is enlarged, and under certain treatments the chromosomes are over-contracted while under others they appear to be deficient in deoxyribonucleic acid. Until cytochemical knowledge of the changes involved in normal mitosis is more clearly defined, it would seem premature to attempt to interpret these effects in detail. It appears certain, however, that M.C.P.A. profoundly modifies the nucleic acid cycle in mitosis, and since Caspersson (1946) has shown that the ribonucleic acids are intimately connected with protein synthesis in the cell, this in turn may mean that the protein metabolism of M.C.P.A.-treated cells is also disturbed.

Many of the effects induced by M.C.P.A. and X-rays are also reported (Koller, 1946) to be produced by mustard gas. For this compound Koller has suggested that the chromatid breakages occur chiefly in regions which are lacking the deoxyribonucleic charge, and this also appears to hold good for the action of M.C.P.A.

Mustard gas has been shown (Dixon and Needham, 1946) specifically to inhibit hexokinase and pyruvate oxidase, and this suggests that mitotic abnormalities may be the result of interference with enzyme action. It would therefore be of interest to determine whether or not M.C.P.A. influences the activity of these and other enzyme systems.

One further effect which has not yet been discussed is the budding and

extrusion of chromatin from the nucleus into the cytoplasm. This phenomenon has been described as occurring in various tumours (Ludford, 1925; Horning and Richardson, 1930; Horning and Miller, 1930). This 'chromidial extrusion' is said to become more advanced with increasing malignancy and is undoubtedly the result of an unbalanced nucleic acid cycle.

A similar effect has also been described by Discombe (1946), in certain leucocytes of mice and of men, in both normal and diseased animals. He suggests that 'extrusion occurs in a cell which is at the height of its physiological powers, but is incapable of further division'. This description may well be applied to M.C.P.A.-treated cells also.

Many of the other abnormalities described in this paper have been reported to occur in certain tumours (Koller, 1943). Since X-rays can both induce and inhibit tumour growth, according to the dosage employed, and since M.C.P.A. causes proliferation of certain plant tissues while inhibiting the growth of others, it would appear that some common factor may be involved in all these effects.

SUMMARY

1. Root-tips of onion (*Allium cepa*) were immersed in (sodium salt of 4-chloro-2-methylphenoxyacetic acid) solutions of 20 to 2,500 p.p.m. concentration for periods varying from $\frac{1}{2}$ to 48 hours and were fixed either immediately on completion of the treatment or after being kept for a further 24-72 hours in water. Preparations were made by the Feulgen root-squash method.

2. Effects of M.C.P.A. action which were visible immediately after treatment at moderate concentrations included over-contraction, swelling and stickiness of chromosomes; chromatin bridges at anaphase with resulting micronuclei; and enlarged nucleoli. At the highest concentrations Feulgen-positive buds were extruded from the nuclei while numerous granules of similar material were present in the cytoplasm and the nuclei stained feebly with leucobasic fuchsin.

3. Many of these effects were also seen in root-tips which had been kept in water for 24-72 hours after M.C.P.A. treatment, but in addition, the effects of chromatid breakages, sometimes followed by interchange, were seen, e.g. acentric fragments, dicentric chromosomes, chromatin bridges (not sticky), micronuclei, and irregular metaphase configurations.

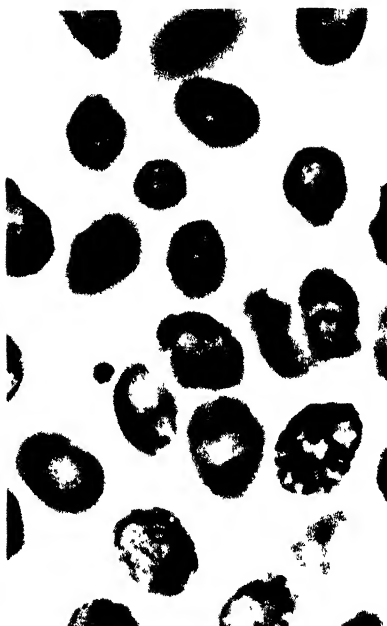
4. The similarity of the effects of M.C.P.A. to those of X-irradiation and mustard gas, and to conditions found in some neoplasms is discussed in relation to the nucleic acid cycle, and the possibility of some common factor linking all these cases is considered.

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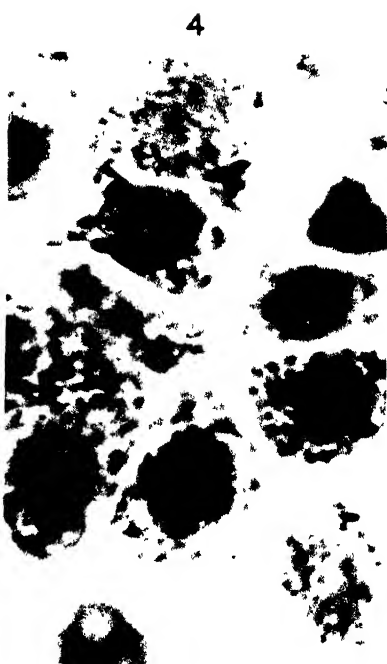
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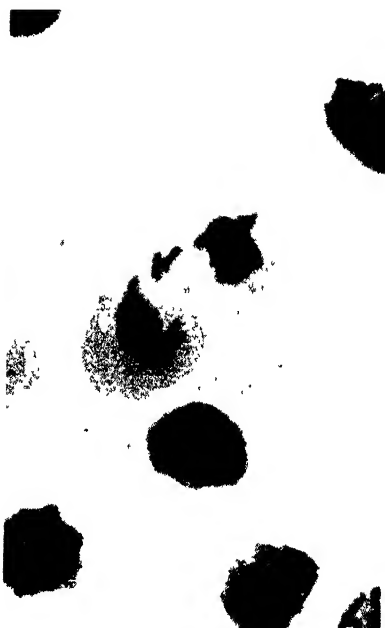
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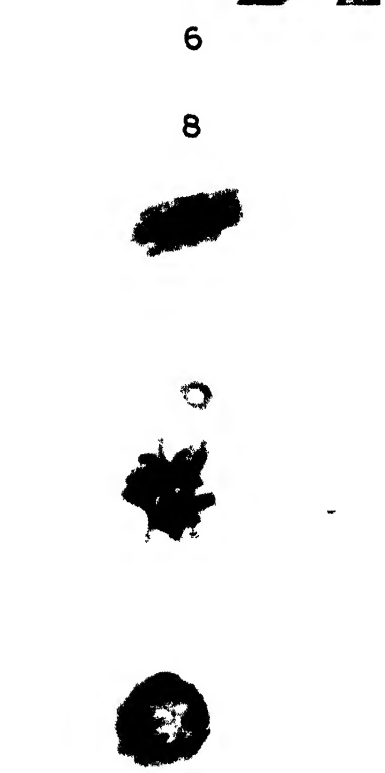
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EXPLANATION OF PLATES I AND II

Illustrating D. Doxey and A. Rhodes' paper on 'The Effect of the Plant Growth-regulator 4-Chloro-2-methylphenoxyacetic Acid on Mitosis in the Onion (*Allium cepa*)'.

All preparations made by the Feulgen root-squash method. All figures from photomicrographs enlarged approximately $2\times$, giving a magnification on the plate of $1,000\times$.

PLATE I

- Fig. 1. Chromatin bridges at anaphase ('sticky bridges').
- Fig. 2. Micronucleus among normal-sized nuclei.
- Fig. 3. Extrusion of Feulgen-positive buds from interphase nuclei.
- Fig. 4. Feulgen-positive granules in cytoplasm.

PLATE II

Fig. 5. Paired chromosomes showing normal degree of metaphase contraction and absence of 'stickiness'.

Fig. 6. Acentric fragments, probably resulting from isochromatid break, lagging on the equatorial plate.

Fig. 7. Dicentric fragment, probably resulting from reunion of non-sister chromatids after breakage.

Fig. 8. Acentric fragment ('ring fragment'), probably resulting from reunion of distal portion of sister chromatids following isochromatid break.

The Effect of Manganese on Carbon Assimilation in the Potato Plant as determined by a Modified Half-leaf Method

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With four Figures in the Text

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INTRODUCTION

THE present work deals with the estimation of carbon assimilation by the potato plant in the field in relation to levels of manganese nutrition.

It has been well known for many years that manganese has a profound effect on plant growth, but little or no systematic inquiry concerning its physiological action appears to have been carried out until the last decade. McHargue

(1926) found less starch and sugar in the 'chlorotic' leaves of manganese-deficient plants as compared with similar leaves on healthy plants, while Miller (1933) found that the addition of manganese to deficient tomato plants more than doubled their sugar contents in 9 days. These results naturally led to the suggestion that manganese might act directly upon the photosynthetic system. Gerretsen (1937), in a comprehensive paper on manganese deficiency in oats, claims to have found assimilation in leaves of manganese-deficient plants to be only half that of normal plants, but no experimental evidence is presented in support of this assertion. Richter and Vassilieva (1941), working in U.S.S.R. with sunflower, kok-saghyz, Perilla, *Vicia faba*, and Hydrangea, grown in soil supplied with a complete fertilizer, claim to have obtained increases in carbon dioxide uptake varying from 12 to 120 per cent. after spraying the leaves with solutions (0.0001 to 0.02 per cent.) of KI, H_3BO_3 , KMnO_4 , or ZnSO_4 , as compared with controls sprayed with distilled water. The leaves were detached at intervals for assimilation measurements and it is claimed that the stimulation persisted for as long as 10 days after the initial spray treatment.

Reuther and Burrows (1942) describe their work on the photosynthetic activity of 'frenched' (manganese-deficient) tung tree leaves in considerable detail. Pairs of shoots showing similar degrees of 'frenching' were selected, and the leaves of one shoot of each pair dipped in a solution of 1 per cent. $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ plus 0.5 per cent. CaCO_3 . Direct measurements of carbon dioxide uptake in the field were made on comparable leaves on each pair, using a Heinicke cup method, at intervals after treatment. Some significant increases in carbon dioxide assimilated by treated leaves were obtained in two out of five series of determinations. These results will be examined later in this paper.

DETERMINATION OF 'CARBON ASSIMILATION' UNDER FIELD CONDITIONS

The most accurate method of determining carbon assimilation is undoubtedly the continuous gas-flow method originated by Kreusler (1885), extensively developed by F. F. Blackman (Blackman and Matthaei, 1905), and first used on attached leaves in this Institute by Chinoy (1935). The method, however, involves considerable elaboration of apparatus if it is to be carried out satisfactorily, and for this reason it was thought advisable to concentrate on the estimation of 'apparent' assimilation by direct determination of the increase in dry matter produced during the day.

In the present investigation use was made of the Roach leaf-stalk injection technique (Roach and Roberts, 1945) to increase artificially the manganese level in one or more leaflets growing on manganese-deficient plants, thus enabling different manganese levels to be compared in the same leaf. The various methods developed for determining dry-weight changes in leaves or leaf parts were therefore critically examined.

The earliest and most widely used procedure is the half-leaf method introduced by Sachs (1884), of which all others are modifications. This depends

on the fact that two halves of any one leaf about the midrib usually exhibit a greater degree of physiological similarity than any two separate leaves. At the beginning of an experiment one half-lamina is cut away close to the midrib and dried. At the end of the experimental period the other half-lamina is similarly treated. Provided sufficient replicates are taken, the difference in dry weight between the two series is a valid measure of the increase in dry weight during the experimental period. In this basic form no information is available whereby the data can be reduced to an area basis, and various modifications have been introduced with this end in view. Sachs himself employed a template to enable a piece of definite area to be cut out of each detached half-lamina, while Ganong (1908) introduced a leaf-punch whereby a piece of known area was cleanly removed from the centre of the half-lamina at each sampling.

Thoday (1909) drew attention to the fact that a leaf may change in area by as much as 5 per cent. during the course of an experiment, and showed that this serious source of error could be avoided if the area to be sampled was marked on both halves of the leaf at the outset with a rubber stamp. This method disregards the deleterious effect of the aniline dyes used and the crushing of the tissues resulting from the mechanical pressure of the stamp.

Although never used by him, Sachs (1884) also suggested the twin-leaf method in which, of two opposite leaves or leaflets, one is sampled at the beginning and the other at the end of the experimental period. Using this method Denny (1930) found that the mean deviation between the dry weights of opposite pairs of leaves sampled simultaneously lay between 4 and 6 per cent. Watson (1936) found that the error using the twin-leaf method for potato leaflets was 11.9 per cent. whereas by random sampling it was 59.3 per cent. Since potato leaves were used in this work it was clear that these methods were subject to large errors and therefore required improving.

The method adopted in the present work owes something to each of the above, since paired leaflets were used and sampled according to a modified half-leaf procedure. It differs, however, in that not only were the areas of the portions removed accurately determined at each sampling, but that other data were collected so that a direct correction for any change in area during the course of the experiment was possible, enabling all results to be expressed on a true area basis.

EXPERIMENTAL METHODS

I. Material and cultural details

Since the primary object of the experiments described herein was to investigate the influence of manganese on carbon assimilation, it was obviously desirable to work with tissues deficient in manganese to ensure that any increase in the manganese level experimentally occasioned might have the maximum effect. The cultural technique described below was accordingly designed, and its success will be evident when the manganese analyses of the experimental leaflets are discussed.

Scotch certified Majestic seed potatoes (Sutton & Sons) were used throughout and were grown in soil, in which manganese-deficiency symptoms are readily developed, from the lake-bed delta at East Malling Research Station. This soil is an alluvial deposit containing considerable calcium carbonate and humus, and has a pH of about 8.0. During 1947 plants were grown both in pots and in the field on the lake-bed delta itself, while in 1946 plants were grown in the field only.

For pot culture a total of some 300 9-in. and 10-in. pots were used. To prevent any supply of manganese from the earthenware a coating of 'Bituros' solution (Wales Dove Bitumastic Ltd.) was applied internally. Sprouted tubers, graded between 50–70 g. per tuber, were planted singly in the pots in sieved soil from the lake-bed delta and first watered on May 14. For efficient drainage a layer of well-washed flints was placed in the bottom of each pot. The number of shoots per pot was limited to three. The plants were grown out of doors and watered at intervals as required. Water of pH 8.0–8.5, drawn from the stream flowing into the lake-bed delta, was used for this purpose, since it was evident that the poor availability of manganese in this soil was principally due to the maintenance of alkaline conditions. The water was carted to the pot site and stored till required in a large tank coated with 'Bituros'.

Field plantings in 1947 comprised some 14 rows approximately 30 yds. long and spaced 4 ft. apart on a small plot situated in the centre of the manganese-deficiency area of the lake-bed delta. The tubers, all above 70 g., were planted 3 ft. apart on May 21. Normal agricultural practice was used throughout. Similar plantings on the same plot were made in 1946.

II. *Use of the 'Roach' injection technique to raise the leaf manganese level*

Roach and Roberts (1945) have shown that if the petiole of a leaflet half-way along a potato leaf is immersed in an aqueous solution the leaflets above and below the point of injection become permeated. The extent of the affected area is indicated approximately in Fig. 1. As injection of Roach's diagnostic solution for manganese containing 0.025 per cent. MnSO_4 + 0.025 per cent. (by volume) of H_2SO_4 had already produced marked visual responses in potatoes grown on the lake-bed delta, it was felt that here was a convenient technique for controlling the manganese level. Thus, if manganese is injected at the point shown, the manganese level in the leaflet *AB* is raised while the opposite leaflet *CD* of this pair forms a control. One drawback of this technique is that the permeation is somewhat heavier on the proximal than on the distal side of the injected leaflet, as will appear later. However, in spite of this defect, the distal pair of leaflets was used.

The Roach injection technique is so well known that it will not be further described here. Attempts were made to standardize the amount of manganese injected by relating the duration of injection to the amount of evaporation taking place from a porous pot atmometer. Field experiments showed no apparent correlation and the method was abandoned. In the event, injection

tubes containing about 1 ml. of solution were left in position for a convenient time and only those leaves which had taken up approximately the same amount (c. 0.5 ml.) accepted as satisfactory.

The question of what time-interval should be allowed to elapse after injection before carrying out an assimilation experiment was a difficult one. The effect, if any, of increased manganese was likely to be small, so that it was important to catch it at its peak if at all possible. Preliminary experiments indicated that the effect was on the decrease 7 days after injection. Failing further information, it was accordingly decided that all experiments during 1947 should take place on the 4th day after injection and thus be comparable among themselves, rather than vary the time-interval in the hope of finding the optimum.

As twelve pairs of leaflets were required for each experiment it was found necessary to inject at least twice this number of leaves in order to allow for failures or subsequent loss through damage. The 3rd or 4th leaf from the apex on a shoot with an active growing-point was selected for this purpose, a different plant being used for each injection. Among the leaves selected the injection points were randomized either on the right- or left-hand side of the petiole in equal numbers. Satisfactorily injected leaves were re-examined the evening before an experiment, and six each of undamaged right-hand and left-hand injected leaves chosen at random. Any visual responses were also noted at the same time.

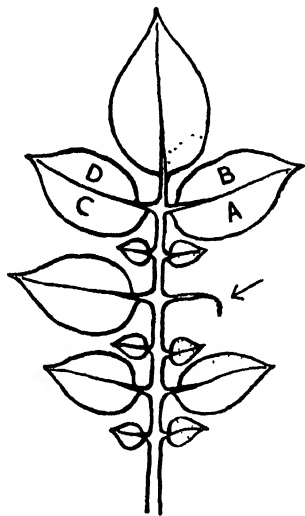


FIG. 1. Potato leaf after injection. The approximate extent of the permeated area is indicated by dotting. The point of injection is marked by an arrow.

III. Determination of leaf areas

The main difficulties in developing the half-leaflet method were due to the unsymmetrical shape of the leaflets and, as this was not avoided by stamping areas on the laminae or punching sections out, the need for an accurate method of determining the areas of the half-leaflets *in situ* became imperative.

Several methods of determining leaf areas in the field were tried, including the spray printing method of Bolas, since used by Goodall (1947). This method, however, suffers from the disadvantage that it cannot be used in wet weather, and furthermore does not permit the position of the midrib to be recorded. In the method finally adopted the areas were traced in pencil directly on to lightly oiled ground-glass plates held in a special holder constructed for the purpose. With this apparatus tracing could, if necessary, be successfully carried on in wet weather, while it was a simple matter to mark in the position of the midrib or any other features required.

(a) *The leaf tracing apparatus.* This is shown drawn in perspective in

Fig. 2. The frame (F) is constructed throughout from angle brass and takes ground-glass plates (G) measuring 3 in. \times 4 in. made from very thin glass (old spectrographic plates). During tracing the leaf is held directly against the smooth side of the glass plate by the rectangular rubber bag (R) so that parallax is reduced to a minimum. This pneumatic bag consists of the valve

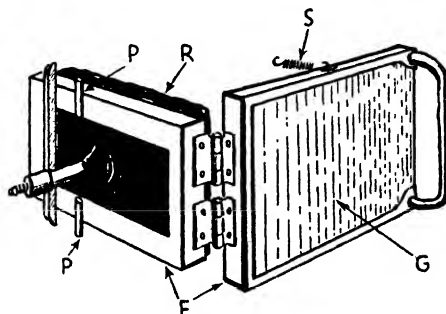


FIG. 2. Leaf tracing apparatus. For explanation see text

portion of a 2 in. \times 1 $\frac{3}{4}$ in. cycle inner tube with a suitably shaped former pushed inside before the ends are sealed. The two parts of the frame are hinged together at the back, the plate and bag being kept in contact by means of two small springs (s) which hook over projections (P) on the lower half of the frame. In operation the top part of the apparatus is raised with the first finger, a new plate slid in, and the leaf placed in position on top of the bag with the other hand. On lowering the top the air pressure in the bag flattens the leaf against the underside of the plate without damaging the leaf tissue. The leaf is then traced directly on to the oiled ground surface of the plate, the top again raised with the finger, and the leaf released unharmed.

(b) *Determination of the areas of the tracings.* By reversing the position of the plate in the holder after taking one tracing it was usually possible to make a second one on the other end of the plate. Both the number of the leaf sampled and the designation letter of the particular tracing were written on the plate at the time of sampling. On this basis a total of five plates was required for each leaf sampled, so that some sixty plates were used for an experiment involving twelve replicates.

As soon as convenient after an experiment these area tracings were printed off in batches of twelve on to standard 'blue-print' paper, using a suitable sized printing frame. Three sheets were printed from each batch of plates, two being used for area determinations, while the third served as a permanent record. A known area was also printed on each sheet at the same time to enable correction to be made for any shrinkage of the blue-print paper during processing. A newly minted halfpenny, diameter one inch (area = 6.45 cm.²) proved very convenient for this purpose.

The completed prints were now cut out with scissors and the areas determined by weighing on a torsion balance (sensitivity ± 0.5 mg.), two replicates

being used for each individual area determination. In cutting out care was taken to cut along the inside edge of all boundary lines since the original tracings had been made on this basis.

(c) *The different area tracings and their uses.* The various tracings taken from the pair of leaflets *AB* and *CD* of Fig. 1 are shown set out in order in

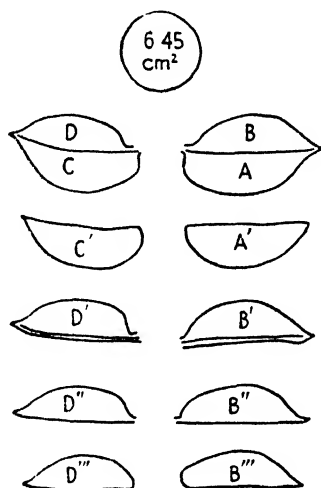


FIG. 3 The complete set of area tracings from the pair of leaflets *AB* and *CD* of Fig. 1. For further explanation see text.

Fig. 3. Particulars of these tracings in relation to the designations used and marked on the drawings are given below:

Designation of tracing.		Relation of tracing to sampling procedure.
Mn injected leaflet	Control leaflet.	
<i>AB</i>	<i>CD</i>	Initial tracing of whole leaflet.
<i>A'</i>	<i>C'</i>	Half-lamina after cutting off at 1st sampling. (Morning sample.)
<i>B'</i>	<i>D'</i>	Half-lamina plus midrib remaining on plant after 1st sampling. Position of midrib marked.
<i>B''</i>	<i>D''</i>	<i>B'</i> and <i>D'</i> retraced to midrib at 2nd sampling. (Evening sample.)
<i>B'''</i>	<i>D'''</i>	Half-lamina without midrib after cutting off at 2nd sampling.

Considering only one leaflet it will be seen that:

- (i) *A'* and *B'''* give the areas of the sampled material *at the time of sampling*.
- (ii) *AB* and *A' + B'* afford one estimate of the error of the method.
- (iii) *B'* cut for a second time along the midrib and *B''* afford an estimate of the change in area of half-lamina *B* during the experiment.
- (iv) A further estimate of error can be obtained by cutting *AB* along the midrib and comparing *B* with *B'* cut as in (iii).

This apparently elaborate technique is, in fact, quite practicable, taking less than 10 minutes per plant at the first sampling and less than 5 at the second.

IV. *Determination of fresh and dry weights*

(a) *Fresh weights.* The half-laminae taken at each sampling were cut off close to the midrib with a scalpel and, after the areas had been recorded, rapidly transferred to weighed and stoppered tubes. These were then brought into the laboratory and the fresh weights determined as soon as possible to the nearest milligram.

(b) *Dry weights.* As soon as the initial weighing had been completed each half-lamina was removed from its tube, fixed on a fine wire hook (tare 40.0 mg.), and transferred to the drying apparatus. This was constructed in the laboratory and is based on the principle of a rapid free flow of heated air past the material being dried. Two one-kilowatt electric fire elements, connected in parallel, are mounted facing each other about 6 in. apart across two bricks. The open ends of the elements are filled in with asbestos sheet so that air is drawn in between the bricks, passes between the fire elements, where it becomes heated and rises through a cylindrical chimney mounted above these elements. As it passes up, the heated air flows over the samples which are suspended by their hooks from stout wires strung across the top of the chimney. Drying temperature is controlled by means of a compound bar, mounted on the side of the chimney at the same height as the suspended samples. This bar switches the current to the heating elements through a 'Sun-Vic' relay. A neon lamp is connected in parallel with the elements as an indicator.

Tests carried out with this apparatus showed that potato-leaf samples could be dried down to constant weight at 60° C. within an hour. The dried material, so produced, showed almost no loss of colour and could be easily reduced to a fine powder with comparatively little grinding. In practice drying of samples was usually continued for about 2 hours.

As the dry weights of some of the half-laminae samples proved to be sensibly less than 10 mg. it was obviously essential, if a reasonable standard of accuracy was to be achieved, that these weights should be correctly determined to at least 0.1 mg. Weighing to this degree of accuracy on an ordinary chemical balance is a tedious process and, even if considerable time is taken over each operation, there usually remains some uncertainty regarding the last figure of the weight. The present experiments called for some forty-eight separate weighings each, so that both on account of speed and sensitivity the use of a chemical balance appeared impracticable.

Since only a limited range of weights had to be determined, use of a spring torsion balance appeared possible, for under these conditions both speed and accuracy are easily obtainable. No suitable instrument covering the required range, however, proved to be available in commerce and therefore a simple balance was made in the laboratory. This instrument, the construction of which has already been described (Portsmouth, 1948), has a range of more than 150 mg. with an absolute sensitivity of 0.07 mg. The balance was used

continually throughout 1947 without trouble. A complete weighing operation takes about a minute.

THE ERRORS OF THE METHOD

I. Comparison with Sachs's half-leaf method

A study of the experimental technique using eight uninjected leaves was made in 1946. Though no attempt was made to estimate assimilation, the normal procedure was followed so that the 'evening' sample was taken immediately after the 'morning' sample. The complete data of this trial experiment are given in Table I. A number of variations between the dry weights of

TABLE I
Trial Experiment, August 23, 1946

(1) Pair No.	(2) Half-lamina.	(3) Position.*	(4) Dry wt. (mg.).	(5) Area.	(6) D.W./cm. ² (mg.).
1	A	RR	42	7.70	5.45
	B	RL	34	5.65	6.02
	C	LL	40	6.95	5.75
	D	LR	32	5.60	5.72
2	A	LR	15	3.25	4.62
	B	LL	21	4.20	4.99
	C	RL	17	3.40	5.00
	D	RR	23	4.75	4.84
3	A	RL	19	3.75	5.07
	B	RR	24	5.05	4.76
	C	LR	17	3.60	4.73
	D	LL	21	4.65	4.52
4	A	RR	47	9.30	5.06
	B	RL	31	6.70	4.63
	C	LL	42	8.45	4.97
	D	LR	30	6.05	4.96
5	A	RL	13	3.40	3.82
	B	RR	19	4.90	3.88
	C	LR	12	3.00	4.00
	D	LL	18	4.45	4.04
6	A	LR	16	4.40	3.63
	B	LL	18	5.00	3.70
	C	RL	16	4.65	3.44
	D	RR	22	6.35	3.46
7	A	LL	25	6.60	3.79
	B	LR	18	4.90	3.68
	C	RR	29	7.50	3.86
	D	RL	22	5.85	3.76
8	A	LL	22	5.05	4.35
	B	LR	18	3.80	4.73
	C	RR	26	5.50	4.72
	D	RL	20	4.25	4.71

* Note. Position of half-laminae sampled are indicated as follows:

LL = Left-hand leaflet, proximal half-lamina.

LR = Left-hand leaflet, distal half-lamina.

RL = Right-hand leaflet, distal half-lamina.

RR = Right-hand leaflet, proximal half-lamina.

All leaves viewed from above from plant axis.

half-laminae and their dry weights per unit area are implicit in these data. Further, these variations can be adjusted, using the method of co-variance on the other half-laminae, thus providing statistical comparison with Sachs's method. There being no difference in the treatment of either leaflet of a pair it is possible to obtain a maximum of sixteen sets of comparisons for estimating the 'morning' weight or dry weight per unit area of the 'evening' sample.

Emphasis must be placed at this point on the asymmetry invariably present in potato leaflets, since, as can be seen from the areas in Table I, the proximal half-laminae are always rather larger than the distal half-laminae. The nature of the asymmetry being known in the present case it was possible to divide the data into two groups, in one of which the proximal half-laminae were corrected by the distal and vice versa in the other. The two errors thus obtained were then combined. Adopting this procedure standard errors of 8.14 per cent. for the Sachs's and 6.03 per cent. for the modified half-leaf method are obtained. Since the ratio of the number of replicates needed to obtain equal accuracy with the two methods is proportional to the squares of these errors, this shows that 1.82 times or nearly twice as many replicates are required using the Sachs's method.

The above comparison, however, shows the Sachs's method at its best since the greatest source of error variance, namely, that arising from the asymmetry of the material, has been eliminated. With many types of field experiments it would be impossible to eliminate this variance. Analysing the present data as one group of sixteen the standard errors are 12.67 per cent. for the Sachs's and 5.87 per cent. for the modified half-leaf method, which indicates that 4.66 or nearly five times as many replicates are required using the Sachs's method for the same degree of accuracy.

II. *Error in determination of leaf area*

The figures given in the preceding section comprise all the errors inherent in the method including those resulting from the leaf-area determinations. Fortunately, as shown in an earlier section, two independent methods are available for estimating this error. Considering the first of these, the combined areas of the two prints A' and B' should equal that of the print AB . The differences in the actual values obtained experimentally thus provide a means of estimating error. For this purpose the differences in area between CD and $C' + D'$ are similar in all respects and may therefore be used in the same estimate of error. No special trial was necessary in this connexion as the actual data from the assimilation experiments could be used. Six experiments, comprising 12 replicates each, were actually carried out in 1947. One set of 12 differences for AB and a similar set for CD was therefore available from each experiment, making a total of 12 sets of 12 differences in all. These data were combined in a single analysis of variance from which a standard error of 0.67 per cent. was evaluated for any one set of 12 replicates.

A second estimate of error is given by the differences in area of the two B (or D) half-laminae obtained by cutting the prints AB (or CD) and B' (or D')

along the midrib. Analysis of this data in the same manner yielded a standard error of 0.90 per cent.

Thus, on either method, it would appear that the error resulting from determination of leaf area is rather less than 1 per cent. for any one experiment.

RESULTS

I. General. Visual response to injection

During the 1947 season some 6 experiments, using 12 leaves each, were carried out, 2 on pot plants, the rest on plants growing on the lake-bed delta. The period over which assimilation was measured varied from $4\frac{1}{2}$ to $7\frac{1}{4}$ hours from dawn onwards. Under the hot, dry conditions often prevailing in the field this season the plants were liable to wilt, for which reason in one experiment the period was reduced to $4\frac{1}{2}$ hours. Details of the various experiments are given in Table II.

TABLE II
List of Experiments

Expt. no.	Date of injection.	Date of sampling.	Material.	Approx. period, hrs.	Conditions.
1	26/6/47	30/6/47	Pot plants	6	In the field
2	10/7/47	14/7/47	" "	6	Under glass
3	24/7/47	28/7/47	Lake-bed plants	7	In the field
4	14/8/47	18/8/47	" "	7	" "
5	28/8/47	1/9/47	" "	$4\frac{1}{2}$	" "
6	11/9/47	15/9/47	" "	$7\frac{1}{4}$	" "

It will be noted that expt. 2 was carried out under glass. One of the primary reasons for using pot material was that, in the event of bad weather, the plants could be moved into a greenhouse and injection and sampling carried out under cover. Fortunately this was the only occasion on which this procedure was necessary.

As mentioned earlier, all manganese-injected leaves were examined for visual responses on the evening before their assimilation was measured. The extent of these responses are given in Table III.

TABLE III
Visual Response to Manganese Injection

Expt. no.	No. of responses noted.	Percentage of plants showing response.	Character of responses.
1	5	21	Slightly increased greenness
2	3	12.5	" " " "
3	18	69	Very "marked." Increased greenness of injected leaflets. Control leaflets developed necrotic spots after injection
4	0	0	Nil
5	3	12.5	Very slight increase in greenness
6	3	12.5	" " " "

The very marked responses observed in the case of expt. 3 are particularly interesting in view of the fact that this experiment was the only one in which what is evidently an anomalous result was obtained for the assimilation determinations. That these leaves were on the verge of death from manganese starvation is evident from the subsequent analyses. In fact these shoots turned brown and died off, except for the injected leaflets which still remained green, within a few days of the conclusion of the experiment.

II. *Fresh weight per unit area*

Values of this ratio, expressed in milligrams per square centimetre and corrected for changes in area during the assimilation period, have been worked out for all the experiments. The figures show no indication of any effect of the manganese treatment and do not warrant waste of space in publication. Such changes in this measure as occur merely reflect the various changes in leaf water content caused by seasonal drift and the hour-to-hour fluctuations in the degree of 'water-strain' prevailing (Portsmouth, 1937).

III. *Dry weight per unit area. Apparent assimilation*

Mean values of this ratio, expressed in milligrams per square centimetre, and corrected for changes in area during the assimilation period, are given for the manganese-injected and control leaflets in Table IV. Apparent assimilations, as measured by the differences in dry weight per unit area between the evening and morning samples, are shown in columns 4 and 7 of this table, while differences in apparent assimilation resulting from manganese injection are given in column 8. The relevant standard errors of all means are included.

TABLE IV
Mean Corrected Dry Weight per Unit Area (mg./sq. cm.)

Expt. no. and date. (1)	Plus Manganese			Control			Effect of Mn. Difference (4)-(7). (8)
	Mean morning. (2)	Mean evening. (3)	Apparent assimilation. (4)	Mean morning. (5)	Mean evening. (6)	Apparent assimilation. (7)	
1 30/6/47	4.261 ± 0.132	4.402 ± 0.090	0.141 ± 0.095	4.116 ± 0.125	4.463 ± 0.117	0.348 ± 0.095	- 0.207 ± 0.085 (<i>P</i> < 0.05)
2 14/7/47	3.654 ± 0.119	3.941 ± 0.100	0.287 ± 0.061	3.523 ± 0.093	3.934 ± 0.107	0.411 ± 0.081	- 0.125 ± 0.129
3 28/7/47	3.554 ± 0.075	4.165 ± 0.139	0.611 ± 0.113	3.303 ± 0.094	3.702 ± 0.099	0.399 ± 0.114	0.212 ± 0.128
4 18/8/47	4.741 ± 0.132	5.393 ± 0.145	0.653 ± 0.155	4.594 ± 0.087	5.514 ± 0.151	0.920 ± 0.144	- 0.268 ± 0.172
5 1/9/47	5.423 ± 0.163	6.057 ± 0.182	0.633 ± 0.152	5.301 ± 0.143	6.070 ± 0.176	0.769 ± 0.147	- 0.136 ± 0.234
6 15/9/47	4.977 ± 0.201	5.455 ± 0.161	0.478 ± 0.110	4.899 ± 0.167	5.530 ± 0.214	0.631 ± 0.100	- 0.153 ± 0.166

With one exception all increases in dry weight per unit area recorded in columns 4 and 7 are obviously highly significant. Only expt. 1, however, shows any significant effect of manganese injection on apparent assimilation,

and this is a decrease. In fact, if expt. 3, which has already been noted as anomalous, is omitted, a decrease in assimilation results from increasing the manganese level in all cases. Combining the differences from the remaining five experiments a mean decrease of 0.178 ± 0.027 mg./cm.² is obtained, which is very highly significant, since $t = 6.687$ for $n = 4$. Expressed on a percentage basis this represents a reduction in apparent assimilation equivalent to 4.02 ± 0.61 per cent. of the initial dry weight per unit area of the morning controls. As the mean increase in dry weight per unit area of the controls is 13.50 ± 1.91 per cent., this represents a decrease in the apparent assimilation measured of almost 30 per cent.

IV. *Initial dry weight per unit area*

One striking result of manganese injection emerges from the data in columns 2 and 5 of Table IV. This is the rather surprising fact that the dry weight per unit area of the manganese-injected leaflets at the beginning of an experiment is consistently higher than that of the controls. The extent of this effect, which is highly significant ($P < 0.01$), is shown in Table V.

TABLE V
Mean Initial Dry Weight per Unit Area (mg./sq. cm.)

(1) Expt. no.	(2) Plus Mn morning.	(3) Control morning.	(4) Difference (2)-(3).	(5) Per cent. difference.
1	4.261	4.116	+0.145	3.52
2	3.654	3.523	+0.131	3.71
3	3.554	3.303	+0.251	7.60
4	4.741	4.594	+0.147	3.20
5	5.423	5.301	+0.122	2.30
6	4.977	4.899	+0.078	1.59
Means	4.435	4.289	+0.146	3.65
S.E.	—	—	± 0.023	± 0.85
t	—	—	6.221	4.277
Value of t for $P = 0.01$			4.032	4.032

V. *Manganese contents*

All the half-laminae sampled in the various experiments were individually analysed for manganese. The method employed was a modification of that adopted by Nicholas (1946) using tetramethyldiaminodiphenylmethane ('tetrabase').

Each half-lamina was ashed separately at a low temperature in an open silica crucible, allowed to cool, and dissolved directly in purified Morgan's Solution. A suitable aliquot of this solution was then made up to 5 ml. with more Morgan's in a 1-in. diameter specimen tube and 0.5 ml. of saturated potassium periodate solution added. The tube was cooled for 1 minute in melting ice, removed from the ice bath, and 0.1 ml. of a 1 per cent. solution of 'tetrabase' in purified acetone added. The blue colour produced with manganese was allowed to develop for 3 minutes and then immediately

matched in a simple colorimeter against an alkaline solution of bromo-thymol blue as standard. All determinations were carried out in duplicate.

No high order of accuracy is claimed for this method, although it would appear that the average error of an analysis is in the neighbourhood of ± 10 per cent., which, in view of the fact that the total manganese to be estimated was often as low as 0.1 gamma, cannot be considered unreasonable.

As mentioned earlier, there is rather heavier permeation of the proximal as compared with the distal half-lamina. This effect was allowed for in the design of the experiments by ensuring that equal numbers of proximal and distal half-laminae were included at each sampling. In Table VI the results of the manganese analyses are presented separately for the two groups of half-laminae, thus enabling the extent of this effect to be seen.

TABLE VI
Mean Manganese Contents in Parts per Million on the Dry Weight

Expt. no. (1)	Mn injected leaflets		Control leaflets		Mean of all injected half- laminac. (6)	Mean of all control half- laminac. (7)	Ratio injected/ control. (8)
	Proximal half- laminac. (2)	Distal half- laminac. (3)	Proximal half- laminac. (4)	Distal half- laminac. (5)			
1	258 ± 28	89 ± 8	29 ± 2	30 ± 2	173.5	29.5	5.88
2	76 ± 4	78 ± 15	24 ± 2	23 ± 1	77	23.5	3.28
3	86 ± 11	20 ± 2	6 ± 0	6 ± 0	53	6	8.83
4	111 ± 11	47 ± 6	11 ± 1	13 ± 1	79	12	6.58
5	101 ± 12	52 ± 8	14 ± 2	14 ± 2	76.5	14	5.47
6	182 ± 18	73 ± 5	19 ± 3	17 ± 2	127.5	18	7.08

The close agreement between the analyses of the two halves of the control leaflets and their small standard errors is satisfactory evidence of the reliability of the analytical procedure. It was to be expected that the errors for the injected leaflets would be larger since they also include the variability in the amount of manganese solution taken up by the different leaves.

That the injection method has successfully increased the manganese content of both halves of the experimental leaflet is obvious from the figures. The extent of this increase in relation to the mean manganese level of the control is set out in column 8 of the table.

DISCUSSION

I. *Manganese levels in the leaves*

There can be little doubt that the manganese contents of the control leaflets, shown in columns 4 and 5 of Table VI, were deficiency levels, since visible symptoms, usually regarded as characteristic of manganese deficiency, were

well in evidence long before the end of the main growing period. Later in the season development of symptoms became much more severe in the lake-bed delta than in the pots, which parallel experiments indicated were limited by nitrogen starvation. As already pointed out, the manganese levels in expt. 3 were so low that these plants were on the verge of death from manganese starvation. It may be noted in this case that the manganese figure of 6 p.p.m. (parts per million) found in the dry material corresponds to less than 1 p.p.m. in the living plant.

Injection of manganese produced visible improvement in leaf appearance in five experiments out of six. This improvement was maintained for many weeks after injection, and in no case was any injury apparent. Consideration of the wide range of manganese contents found in the literature for healthy plants of various species confirms that in no cases were toxic manganese levels reached.

II. *Apparent assimilation*

In expt. 3 the manganese level was so low that the leaves were rapidly becoming moribund. In fact, this process was so far advanced that in the interval between injection and the carrying out of the assimilation determinations the uninjected leaves developed considerable necrotic areas. Manganese injection not only inhibited this process but restored the healthy appearance of the whole leaflet. It is obvious, therefore, that in this case comparison was being made between dissimilar areas of assimilating tissue. It was accordingly decided to reject this experiment from the series.

Consideration of the remaining five experiments leads to the conclusion that increasing the manganese level resulted in a significant decrease in apparent assimilation as measured. This effect might be explained in a number of ways, such as:

- (i) A real decrease in carbon assimilation.
- (ii) An increase in respiration.
- (iii) An increase in translocation rate.
- (iv) An increase in rate of leaf expansion.

The last possibility is ruled out by the fact that area measurements have been made at all stages, so attention must necessarily be confined to the other three. Knowledge of the relative assimilation rates attained in the various experiments is of importance here, so values of this measure, calculated from the experimental data, are presented in Table VII.

Two interesting facts emerge from this table. First, the apparent assimilation rate of the control leaflets in expt. 3 is the lowest rate found, thus confirming the previous conclusion with regard to the morbid nature of this material. Secondly, although there is considerable variation in the relative assimilation rate between the different experiments, the decrease due to manganese injection varies much less widely, about a mean value of approximately 3 mg. per square dm. per hour. This value is roughly the same order as would be expected for leaf respiration (e.g. *Pelargonium* leaves,

Portsmouth, 1934) and so could only be accounted for by, at least, a doubling of the respiration rate.

TABLE VII

Apparent Assimilation calculated as Milligrams Increase in Dry Matter per Square Decimetre of Leaf Surface per Hour

Expt. no. (1)	Mn injected leaflets. (2)	Control leaflets. (3)	Decrease due to Mn injection. (4)
1	2.35	5.80	3.45
2	4.78	6.85	2.07
3	8.73	5.70	(-3.03)
4	9.33	13.13	3.80
5	14.06	17.10	3.04
6	6.59	8.71	2.12

Recent work by Kornberg, Ochoa, and Mehler (1948) has shown that manganese is necessary for the activity of the oxalosuccinic and oxalacetic acid decarboxylase systems, both of which are stated by Baldwin (1947) to form part of the normal aerobic respiration cycle. On these grounds, therefore, a real increase in the respiration rate with manganese appears possible, although it seems unlikely that the increase will be large enough to account for all of the observed effect.

That manganese acts by bringing about a reduction in the real rate of carbon assimilation appears improbable in view of the numerous cases of increased growth and yield in various field crops reported to result from manganese treatment. Even a small-scale spraying experiment with manganese sulphate carried out on the potatoes growing in the lake-bed delta plot in 1947 resulted in significant increases in tuber number and yield as shown in Table VIII.

TABLE VIII

Effect of Manganese Spraying on Majestic Potatoes, 1947

	Manganese sprayed.	Controls.	Increase due to Mn.	Significance.
Mean tuber number	17.2	13.6	3.6	$P < 0.05$
			± 1.48	
Mean yield per plant (kg.)	1.616	1.185	0.431	$P < 0.01$
			± 0.125	
Mean weight per tuber (g.)	94.0	87.1	6.9	—

If the above reasoning is valid, increased translocation must remain as one of the most likely causes of the experimental results reported in this paper. Some further slight support is given to this hypothesis by a small-scale pot experiment in which an indication of earlier tuberization as a result of manganese spraying was obtained. It is hoped that, as the result of further experiments, this point can be settled definitely in the near future.

Bolas and Selman (1936) and Goodall (1946), working with young tomato plants, have shown that a large proportion of the assimilates formed are translocated out of the leaf in the light. Thus a small increase in the translo-

cation rate could easily produce an apparent decrease in assimilation. Furthermore, if the law of mass action holds in relation to any of the products of photosynthesis an increase in the rate of removal of these from the leaf will of necessity cause a rise in the photosynthetic rate, which cannot be detected by the method used.

III. *The experiments of Reuther and Burrows (1942)*

The results obtained by these workers, already referred to in the introduction, are reprinted in full in Table IX.

TABLE IX
Carbon Dioxide Assimilation of Frenched Tung Tree Leaves.
(Data from Reuther and Burrows, 1942)

Type of leaf.	No. of replicates.	Mean assimilation in mg./100 cm. ² /hr.		Difference.	Significance.
		Plus Mn.	Control.		
	Period I. June 10 to 24.				
Shoot	18	7.26	7.24	+0.02	N.S.
Basal	18	6.41	6.68	-0.27	N.S.
	Period II. June 25 to July 11.				
Shoot	18	8.68	6.89	+1.79	$P = 0.05$
Basal	19	6.79	7.20	-0.41	N.S.
	Period III. August 4 to 12.				
Shoot	25	7.95	6.31	+1.64	$P = 0.05$

Date of treatment - June 10.

It will be seen that the basal leaves show no increase in assimilation rates as a result of the manganese treatment, but they also showed very few symptoms of deficiency. The shoot leaves, on the other hand, showed marked deficiency symptoms at the outset. As a consequence of the manganese treatment considerable differences in appearance were soon evident, since the treated leaves became very much greener and healthier looking than the controls. A scoring of symptoms 19 days after treatment yielded the following figures for the 'chlorotic index'.

Type of leaf.	Value of chlorotic index for	
	Plus Mn.	Controls.
Shoot	57	205
Basal	19	39

In view of the above it seems probable that the significant increases in assimilation obtained for the treated shoot leaves during the two later periods are better ascribed to a decrease in the amount of actively assimilating tissue in the control leaves, as a result of manganese deficiency, rather than to an actual stimulation of the photosynthetic rate by manganese.

IV. *Increase in initial dry weight per unit area*

The increase in this ratio invariably resulting from manganese injection and given on a percentage basis in column 5 of Table V is shown plotted

against time in Fig. 4. It will be seen that the effect changes quite regularly throughout the season, reaching a maximum at the end of July. Still greater increases, up to 20 per cent. ($P = 0.001$), 10 days after injection, had previously been observed by Bolas (1946), who regarded them as evidence for increased assimilation.

Correlation of the absolute increase in dry weight per square centimetre, given in column 4 of Table V, with the increases in manganese content result-

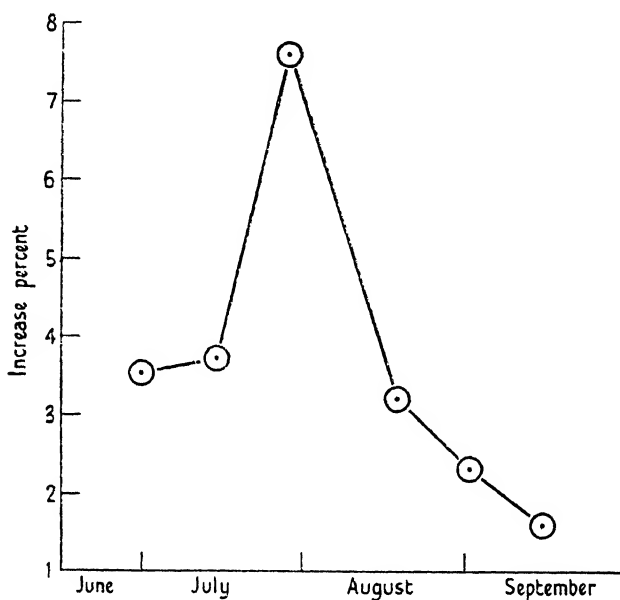


FIG. 4. Graph showing change with time of the initial increase in dry weight per unit area resulting from manganese injection.

ing from injection, and shown as ratios in column 8 of Table VI, provides an indication that the effect of manganese in this connexion may have a quantitative basis. Using the data from all six experiments a total correlation of 0.526 is obtained. If, however, expt. 6 is rejected on account of the probable senescence of the plants at such a late date in the season, the correlation rises to 0.837 and approaches significance. The possible existence of some such relationship as is suggested here would appear to afford further confirmation that no toxic manganese levels were reached after injection.

Explanation of this effect, in the absence of any further information, remains difficult and only tentative suggestions can be advanced. What is beyond dispute is that an increase in dry matter has occurred as the result of increasing the manganese level. Two sites may be postulated for this increase, namely, the cell wall or the cell contents. Comparison of the total areas of the injected and control leaflets recorded at the morning sampling affords no support to the former. The figures are given in Table X, and it will be seen that there is no evidence of any increase in leaf area after injection.

TABLE X
Total Leaflet Areas (sq. cm.)

Expt. no.	Manganese injected.	Control.
1	99.90	103.60
2	80.42	78.71
3	101.25	101.37
4	94.46	96.52
5	87.01	86.20
6	108.56	108.73
Sum	571.60	575.13

Turning to the second possibility, an increase in leaf thickness or in the specific gravity of the cell content must occur, either or both of which could be explained on the basis of an increased protein content. That manganese may play a part in protein synthesis is by no means improbable and has much collateral evidence to support it. Manganese is known to play an essential part in many enzyme systems, a number of which are concerned in nitrogen metabolism. Apart from evidence from enzyme systems, the possibility that manganese is required in some of the later stages of protein synthesis is suggested by some analyses of green and chlorotic *Macadamia* leaves carried out by Guest (1943). He found that the green leaves had more dry matter, more sugars, and more manganese, and less ammonia, amide, and amino nitrogen, than the chlorotic leaves. Further argument is provided by the development of necrotic areas on the leaves in cases of severe manganese deficiency as was observed in expt. 3. These areas appear to arise through actual death of the cytoplasm, so it is reasonable to assume that a process of proteolysis sets in in the absence of manganese.

V. Concluding remarks

Although a decrease in apparent assimilation has been found to occur as a result of raising the manganese level this must not be taken to mean that there has been a decrease in the real assimilation rate. On the face of it such a state of affairs is highly improbable. Thus it is much more likely that the decrease observed can be accounted for as the resultant of (i) an increase in respiration rate, and (ii) an increase in the rate of translocation of assimilates out of the leaf. The increase in dry weight per unit area as a result of raising the manganese level, which is well established, may indicate a dependence of protein synthesis upon manganese, but this will obviously require special investigation.

SUMMARY

1. Techniques for investigating the effect of manganese on the carbon assimilation of potato plants in the field are discussed and an improved half-leaf method of measuring apparent assimilation described.
2. The new method enables asymmetric leaves to be used without loss of

accuracy and, under these conditions, shows a fivefold increase in precision over the original Sachs's half-leaf method.

3. The method depends on the use of a simple and accurate method for determining leaf areas both at the beginning and end of each experiment. The errors of these determinations are less than 1 per cent.

4. Manganese-deficient potato plants were grown and the Roach injection technique used to raise the manganese level of one leaflet of a pair, thus providing a physiologically similar control.

5. Using the methods described it is found that raising the manganese level results in no increase but a significant decrease in *apparent* assimilation. The reasons for this are discussed.

6. In spite of the previous finding a highly significant increase in dry weight per unit area is found to have occurred 4 days after manganese injection. A possible reason for this is suggested.

ACKNOWLEDGEMENTS

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Some Observations on the Receptacles of *Scytothalia dorycarpa*, with Special Reference to the Extrusion of the Oogonia¹

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With four Figures in the Text

MEMBERS of the Fucaceae are found in a variety of habitats in both the northern and the southern hemispheres. The three genera of the northern waters, *Pelvetia*, *Fucus*, and *Ascophyllum*, are all intertidal forms, whilst the majority of the genera of the southern hemisphere, on the other hand, are found growing in submerged habitats.

Scytothalia dorycarpa, one of the latter class, is a specialized and endemic species which occurs off the south coast of Australia and off the coast of Tasmania. It grows on rocks just below low-tide level, where it forms bushy plants about 2 ft. high (Turner, 1811; Harvey, 1858; De Toni, 1895).

The plant consists of a monopodial axis, which bears at its margins leafy laterals, adventitious branches, and fertile receptacles (Fig. 1, A). The axis is flattened with no midrib, and with its growing point in an apical depression protected by the incurved rudiments of the leafy laterals. These leafy laterals taper towards their tips, have strongly decurrent bases, and arise from the margins of the thallus in a distichous series. Adventitious branches which repeat the structure of the main axis arise from its margins at intervals between the leafy laterals.

The receptacles are restricted to special receptacles which arise in linear series from the margins of the axis, in positions supra-axillary to the leafy laterals. The members of any one group of receptacles are about the same age, the youngest groups occurring nearest the apex. These receptacles, when mature, are lanceolate in shape, with a pointed, sterile tip and a rounded attaching base.

The material investigated was collected in February 1920 by Professor Osborn,² from a rocky headland west of Encounter Bay, South Australia. It consisted of the tips of two branches bearing receptacles at all stages of development, including a number of fairly mature ones. The remaining pieces were

¹ This work formed the subject of a thesis for the degree of M.Sc. in the University of London.

² In 1945 Professor Osborn gave this material to Dr. E. M. Delf, who handed it to me for investigation. I should like to take this opportunity of thanking Professor Osborn for collecting and preserving this material. It was fixed in a mixture of formalin, sea-water, and alcohol, and later preserved in 50 per cent. alcohol.

rather fragmentary, and their position relative to the plant as a whole could not be judged. So far as could be seen from the material available, the specimen coincided with the *Fucus dorycarpus* of Turner (1811) and with the *Scytothalia dorycarpa* of Harvey (1858) and De Toni (1895). Agardh separates the genus *Scytothalia* into two species—*S. xiphophora* and *S. dorycarpa*—on the basis of receptacle shape, but in this small amount of material examined there was considerable variation in receptacle shape, so this does not appear to be a constant feature.

The structure of the thallus

At the apex of the thallus is an elongated groove filled with mucilage, and containing at its base a single, large, four-sided cell, confirming that *S. dorycarpa* is one of the Fucaceae (Grüber, 1898; Schmidt, 1938).

The axis is bounded by a single-layered meristoderm of brick-shaped cells with dense granular contents. Within this is a cortex about ten cells in depth, and in the centre is a medulla of cells with rather sparse contents, elongated in the longitudinal direction, and separated by mucilage. The outer cells of the cortex are isodiametric with dense contents and resemble those of the meristoderm, but on passing inwards there is a gradual transition to elongated cells resembling those of the medulla. Consequently there is no sharp boundary between the cortex and the medulla.

No hyphae were visible in the region of the apex, but about 3 cm. behind the apex a few were visible running longitudinally between the cells of the inner cortex. The origin of the hyphae could not be seen, but they were easily detected on account of their thick refractive walls. With increasing distance from the apex more hyphae are produced, and they penetrate progressively towards the centre of the axis, interweaving between the medullary cells as well as between the cortical cells. This increase in hyphal production in the older parts of the axis agrees with the condition in *Fucus* and in *Halidrys* (Fritsch, 1945).

Hyphal production is particularly abundant in the basal part of the axis, where it is rounded and not flattened, and which region is presumed to be the basal-attaching portion of a lateral branch. This pronounced development at such a point is comparable with the condition in *Ascophyllum nodosum*, where there is similar development of hyphae towards the bases of the lateral branches (Oltmanns, 1889).

The leafy laterals have no special initial cell, and their anatomical structure is similar to that of the main axis, but little hyphal development was seen.

No cryptostomata were seen either on the axis or on the leafy laterals.

The anatomy of the receptacles

The receptacles differ very little internally from the rest of the thallus and consist of the same three tissues. The structure of the sterile tip is similar to that of the main axis in the region of the apex, and the fertile region differs from the main axis chiefly in the frequent interruption of the meristoderm

and cortex by the conceptacles. No hyphae were seen in these two regions, but they were developed in great numbers in the rounded attaching portion of the receptacle, which thus shows similarity with the basal portion of the vegetative branch. A further point of similarity is that, in both these regions, where there is a rapid narrowing of the thallus and a change from bilateral to radial symmetry, there is great tangential activity of the meristoderm, which has cut off radial files of cells (cf. Halidrys, Fritsch, 1945).

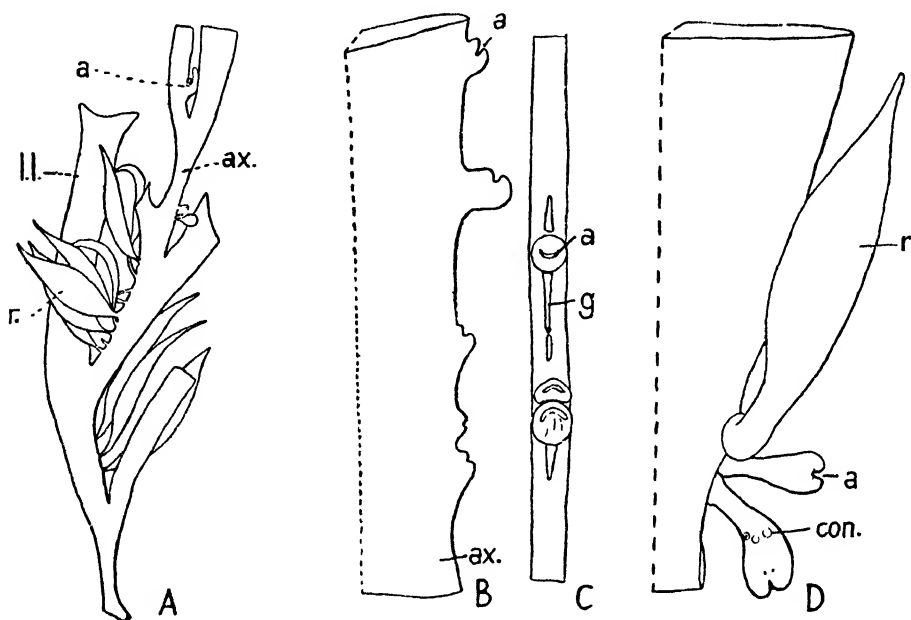


FIG. 1. A. Part of a frond of *Scytothalia dorycarpa*, natural size. B, C, and D. The fertile margin of the axis, showing the development of the receptacles. B, side view; C, end-on view; D, side view, slightly older. (a, indented apex; con, conceptacle; g, marginal groove of the axis; ll., leafy lateral; r, receptacle, conceptacles omitted.)

The development of the receptacles

The receptacles develop in marginal grooves in the axis (Fig. 1, C). When the receptacle first projects beyond the groove it has a deeply indented apex surrounded by one large and one small incurved lobe (Fig. 1, B). This asymmetry is soon lost, but the indented apex is retained (Fig. 1, D) until after the conceptacles are laid down, when it disappears and the sterile tip is produced.

The marginal groove in which the receptacles arise is confined to one margin of the axis, which may be slightly flattened in comparison with the other sterile margin. The fertile margin alternates regularly from one side of the axis to the other in successive 'internodes'.

The groove is lined with a meristoderm continuous with that of the rest of the axis. The cells are characteristically flattened and are apparently mucilage-producing, as the groove is filled with mucilage, which extends into

the centre as a series of caps arising from these cells (Fig. 2, c). There has been considerable tangential activity of the meristoderm in this region, as the cells of the outer cortex are arranged in regular, radiating rows lining the groove.

At intervals along the groove are deeper pits, also filled with mucilage, and containing a single large cell at their bases (Fig. 2, b). Possibly these are the initials of future receptacles, as similar large cells can be seen at the base of the apical depressions of young developing receptacles.

THE CONCEPTACLES

The conceptacles are borne on both sides of the receptacles in acropetal succession, but no definite pattern of arrangement could be seen, such as the regular spiral arrangement demonstrated by Dawson (1940) in *Carpophyllum flexuosum*. They are visible as lighter patches, more or less circular, but sometimes elongated in the direction of the longitudinal axis. The ostiole is usually a circular hole in the centre, but where the conceptacle is elongated, the ostiole is similarly elongated into a slit.

Both male and female conceptacles occur on the same receptacle. In all the material examined—about forty to fifty receptacles—the female were far more numerous than the male, outnumbering them by about ten to one, thus showing a tendency towards economy in the number of spermatozooids produced.

Occasionally hermaphrodite conceptacles were found. In some there were tufts of antheridial hairs amongst the paraphyses separating the oogonia, whilst in others one side of the conceptacle was entirely male and the other entirely female.

The conceptacles are lined with a layer of small cells difficult to distinguish. There are no periphyses guarding the ostiole in either the male or the female conceptacle, but in both the ostiole is lined with small cells similar to those of the meristoderm.

The male conceptacles

The male conceptacles contain antheridial hairs of typically fucalan structure (Fig. 2, d). The hairs arise in tufts from small mounds of cells in the walls of the conceptacles. They are multicellular and much branched, and bear numerous antheridia which have swollen walls when mature. There are no sterile hairs, either separating the antheridial hairs or guarding the ostiole.

The female conceptacles

The female conceptacle (Fig. 2, e) contain oogonia at all stages of development, indicating the production of a succession of ripe oogonia, as in *Fucus*, and not the simultaneous ripening of all the oogonia within the conceptacle, as in *Sargassum Horneri* (Tahara, 1913).

The oogonia are separated by sterile hairs, which arise in tufts from mounds of cells in the walls, in a manner similar to the origin of the

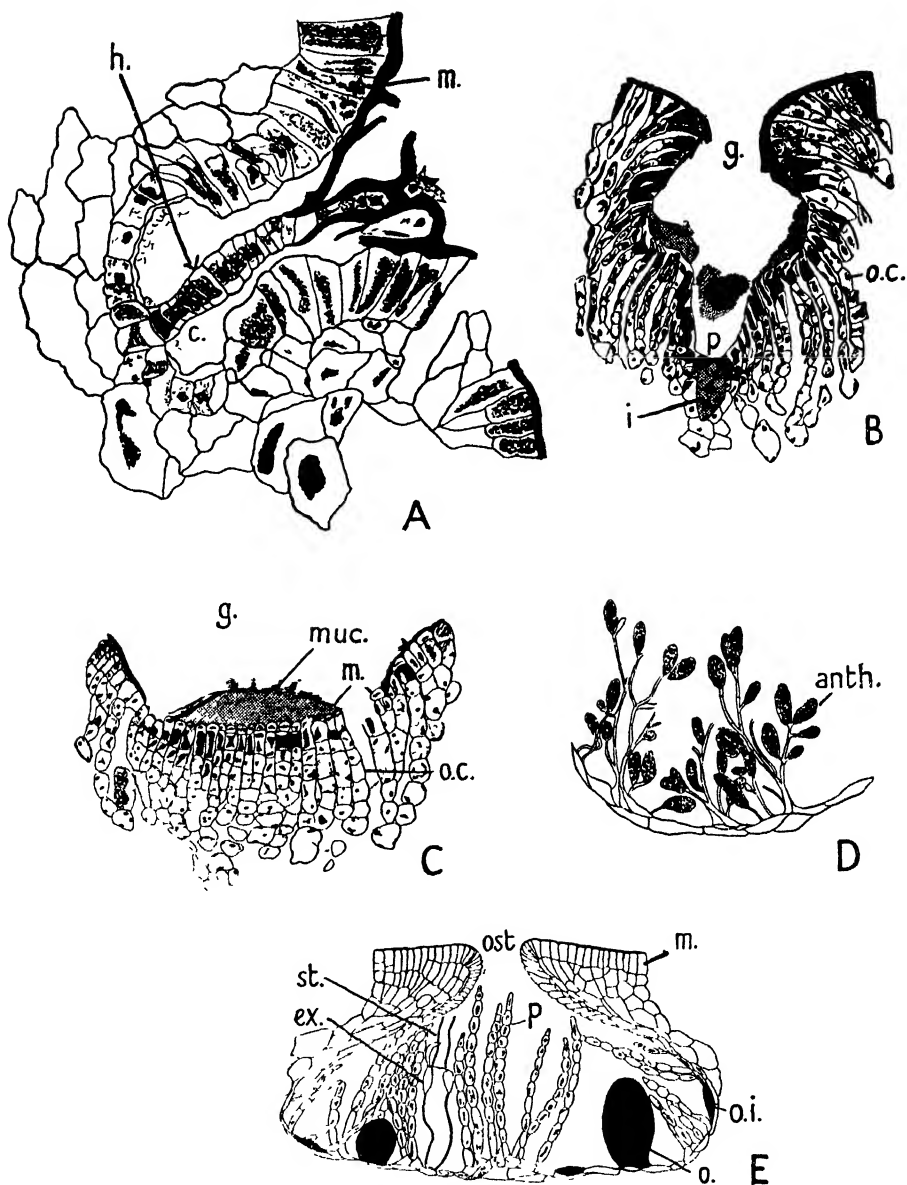


FIG. 2. A. Young conceptacle with a basal ectocarpoid hair (*h.*). B. Transverse section through the marginal groove (*g.*), showing the pit (*p*) containing the initial (*i*) o.c., outer cortex. C. Transverse section of the sterile region of the marginal groove (*m*, meristoderm; *muc*, mucilage). D. Branched antheridial hairs on the wall of the male conceptacle (*anth*, antheridium). E. Female conceptacle in transverse section, slightly diagrammatic (*ex*, exochiton; *o.i.*, oogonial initial; *ost*, ostiole; *p*, paraphysis; *st*, mesochiton stalk).

antheridial hairs. These paraphyses reach almost to the ostiole, but do not project through it.

The paraphyses are all of one kind—unbranched, and composed of barrel-shaped cells with rather swollen walls. There are no traces of any central group of basally growing 'ectocarpoid' hairs, such as those described by Wille (1910) in *Himanthalia lorea*, by Dawson (1941) in *Cystoseira foeniculacea*, and by Delf and Cooke (in press) in *Turbinaria turbinata* (?). A single ectocarpoid hair was seen in the young conceptacle of *S. dorycarpa*, but could not be detected in the mature conceptacle. In the mature conceptacle there are no signs of any branching paraphyses, such as the 'Cladophora-like' paraphyses described by Wille (1910) in *Himanthalia lorea*. Such branching hairs are rare in female conceptacles, and are recorded in *Carpophyllum flexuosum* (Dawson, 1940) and in *Fucus vesiculosus* (Nordhausen, 1910). Branching hairs are found in the female conceptacles of *Durvillea*, but in this case they are fertile, bearing the oogonia (Whitting, 1893; Herriott, 1923). Such branching hairs are similar to the antheridial hairs of the male conceptacles.

Very little mucilage could be seen in the conceptacles, whether free in the cavity of the conceptacle or in association with the paraphyses. This contrasts strongly with the condition in *Fucus*, where there is abundant mucilage in the conceptacle, and also with *Turbinaria*, where the mucilage can be seen as a series of caps extending from the tips of the paraphyses (Delf and Cooke, in press). This absence of mucilage in *S. dorycarpa* may be due to the fact that the material was fixed in a medium containing formalin, which rapidly breaks down mucilage. On the other hand, there was abundant mucilage in the marginal groove, so that this absence from the conceptacle may be real, and possibly correlated with the elaboration of the oogonial wall.

The development of the conceptacle

In the earliest stages found the conceptacle was a narrow slit containing a single hair with a basal meristem (Fig. 2, A). Similar hairs are produced by the divisions of the tongue cells in the juvenile conceptacles of *Bifurcaria tuberculata* (Rees, 1933), in *Carpophyllum flexuosum* (Dawson, 1940), in *C. maschalocarpum* (Delf, 1939), and in *Halidrys dioica* (Nienburg, 1913; Doubt, 1928). Such hairs are not regarded as a usual feature of the Fucaceae, but are more frequent amongst the Sargassaceae. They are reported in *Pelvetia fastigiata* (Nienburg, 1913) and occasionally in *Fucus platycarpus* (Bower, 1880).

In the young conceptacle the lining of the ostiole seemed to be formed from the meristoderm and not from the divisions of the conceptacle initial (Fig. 2, A). In this it resembles *Fucus*, *Ascophyllum*, and *Halidrys*, and differs from *Sargassum*, *Carpophyllum maschalocarpum* (Delf, 1939), and *C. flexuosum* (Dawson, 1940), in which the products of the initial contribute towards the lining of the ostiole.

THE OOGONIUM

The development of the oogonium

The oogonium first becomes visible as a slightly elongated, deeply staining cell in the wall of the conceptacle. This increases in size until it is an ovoid structure $120-40\mu$ in length, projecting into the cavity of the conceptacle. A stalk cell could not be recognized with any certainty.

At this stage the cytoplasmic contents of the oogonium are surrounded by a thin wall which does not stain with gentian violet. The further develop-

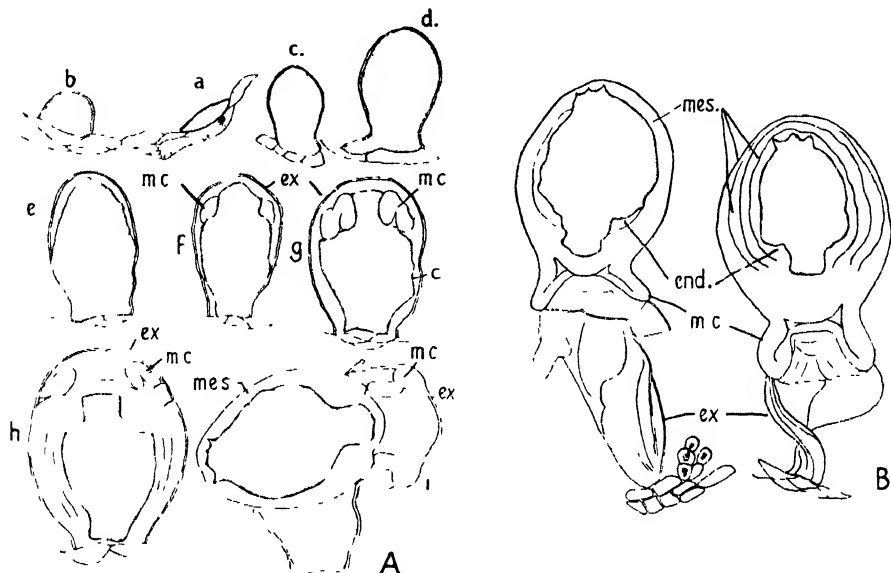


FIG. 3. A. Stages in the development of the oogonium *a-e*, early stages, before the differentiation of the oogonium wall, *f-g*, stages in the development of the mesochiton collar (*mc*); *h*, a more mature oogonium with swollen mesochiton (*mes*) already showing differentiation into three layers, *i*, oogonium with ruptured exochiton (*ex*) with the mesochiton stalk fused to the exochiton *c*, cytoplasmic contents; *end*, endochiton B. Two extruded inverted oocytes attached to the ruptured exochiton.

ment of the oogonium consists in the elaboration of this thin wall into a complex mucilaginous structure composed of several distinct regions. Although this development involves considerable change in the shape of the cytoplasmic contents, there is no appreciable change in their volume.

The first stage in this differentiation consists in the appearance of a thickened ring of material in the apical region (Fig. 3, A, *e*). This ring increases in size, causing the constriction of the cytoplasmic contents of this region, resulting in their characteristic 'amphora' shape. As the ring becomes more sharply differentiated it can be seen to be folded back on itself, giving a typical 'ear-like' appearance in optical section (Fig. 3, A, *f* and *g*).

The entire wall now increases considerably in thickness, owing to the development of a mucilaginous region completely investing the cytoplasmic contents. At first this is only a thin layer, but it becomes progressively thicker,

and by its swelling it forces the thickened collar away from the cytoplasmic contents, and itself extends up the centre of the collar into the space previously occupied by the narrow neck of the cytoplasmic contents (Fig. 3, A, *h*). Its swelling also separates the basal region of the cytoplasmic contents from the oogonial wall and so causes it to become drawn out into several tapering prolongations.

The apical collar appears to be of a tougher consistency than this swollen region and is easily distinguished from it, both by its highly refractive appearance in unstained preparations and by the fact that it stains more readily and more intensely with gentian violet.

This swollen region now becomes differentiated into three concentric lamellae (Fig. 3, A, *h*), and a further fine membrane appears around the cytoplasmic contents (Fig. 3 B, *end*).

Thus the wall of the fully developed oogonium is a highly complex structure, consisting of two membranes, separated by a central mucilaginous region, in which can be distinguished two separate regions—the tougher, folded apical collar, and the very swollen portion entirely surrounding the cytoplasmic contents.

The layered nature of the oogonial wall of *Fucus* was realized by Decaisne and Thuret (1845),¹ who recognized the thin outer layer left behind in the conceptacle after the eggs had been shed enclosed in the swollen portion of the walls. Thuret and Bornet (1878), working on the release of the eggs in *Fucus*, demonstrated the presence of three layers in the oogonial wall, an inner and an outer fine membrane and a central swollen portion. These observations were later confirmed for *Fucus serratus* by Farmer and Williams (1898), who used the terms 'endochite', 'exochite', and 'mesochite', respectively, to describe these layers.

The inner and the outer layers of the oogonial wall of *S. dorycarpa* are comparable both in position and in structure with the endochiton and the exochiton of *Fucus*, but the middle, swollen region of the wall of *S. dorycarpa* is a much more complex structure than the mesochiton of *Fucus*.

Thuret and Bornet (1898) showed that the mesochiton of *Fucus* is not merely a mucilaginous mass, but it is surrounded by a very fine outer membrane, which, on the release of the egg packets, peels off the inner portion of the mesochiton, which then dissolves. No similar outer mesochiton membrane was seen in *S. dorycarpa*, but it may possibly be represented by the apical collar which seems to be firmer than the rest of the mesochiton.

Resühr (1935) describes the mesochiton of *F. vesiculosus* as being still more complex, and as consisting of four layers—two relatively firm membranes separating two jelly layers, the 'mesogallerte' and the 'enterogallerte'. These two jelly layers may be comparable with the three concentric layers of the mesochiton seen in *S. dorycarpa*, but it is not clear in the latter case that these layers are separated by any membrane. I was not able to distinguish any layers in the mesochiton of plants of *F. vesiculosus* collected in the

¹ Cited from Resühr, 1935.

Thames estuary, but this may be due to a difference in salinity of the water, as Resühr found the layers much more difficult to see in plants collected from Kiel than in plants collected off Heligoland, a variation which he ascribes to a difference in salinity. The clearness with which the layers could be seen in *S. dorycarpa* depended to a large extent on the mounting medium. They were hardly visible in dilute glycerine, but very clear in distilled water, and even clearer in potash or alcohol.

Thus, despite its apparently much greater complexity, the central region of the oogonial wall of *S. dorycarpa* may be reasonably considered as mesochiton, comparable with the mesochiton of *Fucus*.

The endochiton of *S. dorycarpa* (Fig. 3, B) often does not become differentiated until after extrusion, as in *Fucus*, and often appears first at the 'neck' end, as in *Marginariella Urvilliana* (Delf, 1937).

Oogonia of similar construction have been described in *Marginariella Urvilliana* (Delf, 1937) and in *M. boryana* (Delf and Hyde, 1936), both of which, like *S. dorycarpa*, are endemic Australasian members of the Fucaceae. All these species possess very swollen oogonial walls with similar apical collars of mesochiton.

The swollen region of the mesochiton of *S. dorycarpa* is very similar, both in structure and in development, to the layer described as 'endochiton' in *M. Urvilliana* (Delf, 1937, Fig. 1c and 3d). After extrusion this 'endochiton' is figured as showing differentiation into three layers (Delf, 1937, Fig. 3g) similar to the three layers in the mesochiton of *S. dorycarpa*. Despite these obvious similarities between these regions I prefer, for reasons already stated, to interpret this layer as mesochiton, and not as endochiton.

The region of the oogonial wall of *M. Urvilliana* described as 'appearing as though full of small droplets' (Delf, 1937, Fig. 1c) is no doubt comparable with the region interpreted as endochiton in *S. dorycarpa*.

The organization of the oosphere

Each oogonium produces a single oosphere, but the time of nuclear maturation and organization of the oosphere could not be determined with any certainty. The onset of nuclear division appeared to coincide with the differentiation of the oogonial wall, with the first two divisions following very closely upon each other. The three nuclear divisions characteristic of the Fucaceae, with the resulting formation of eight nuclei, takes place in *S. dorycarpa*.

Whether the final organization of the uninucleate oosphere takes place before extrusion, or whether the contents are extruded in a multinucleate state, it is not possible to say. The occurrence of large numbers of oogonia with fully differentiated walls, and with still multinucleate contents, suggests that the latter may be the case. But, on the other hand, contents which had been extruded were clearly seen to be uninucleate, suggesting that the organization had taken place either immediately before or during the process of extrusion. Possibly the condition of the nuclei at the time of extrusion may be a

variable feature, the time of extrusion being determined by other factors such as the state of maturity of the oogonial wall or the tidal conditions.

Because of the possibility that the contents are liberated whilst still in a multinucleate state, the term 'oocyte' is used to describe the escaping contents. This term was used by Mitchell (1941) to describe the escaping contents of the oogonium of *Xiphophora chondrophylla*. Tahara speaks of 'oogonium liberation' in *Sargassum Horneri*, but this is unsatisfactory, as the same term is then used to describe both the entire structure and the escaping contents.

There are no signs of any extrusion of the seven supernumerary nuclei, so that the final uninucleate condition appears to be arrived at by their degeneration *in situ*, as in *Sargassum Horneri* (Tahara, 1913), and not by their extrusion as in *Coccophora Langsdorfi* (Tahara, 1929) and *Cystoseira barbata* (Nienburg, 1910).

The mechanism of extrusion

Very little information concerning the mechanism of extrusion of the oocytes could be obtained from a study of the untreated material. Two escaped oocytes were found, still surrounded by the swollen mesochiton, and attached, in an *inverted* position, to the ruptured exochiton by the apical collar of mesochiton (Fig. 3, B). In several other places strands of mucilage were found attached to empty exochitons (Fig. 2, E, *st*).

Attempts were made to induce the extrusion of the oocytes by placing sections containing oogonia in 1 per cent. aqueous potash. This causes a swelling of the walls, which may, in the case of fairly mature oogonia, be sufficient to initiate the liberation of the contents. In considering the results of such experiments it must be borne in mind that these oocytes are dead structures and are far less elastic than in the living condition. Consequently they may not be able to reproduce the living behaviour in every respect. Very similar stages were seen in both the untreated and in the treated material, so probably a fairly good idea of the manner of extrusion in the living plant can be gained from a study of the stages obtained by this treatment.

In some cases a longitudinal split appeared in the exochiton, extending from just below the apical collar to the base of the oogonium (Fig. 4, A, B). The oocyte, surrounded by the mesochiton, began to bulge through this split, followed by the apical collar and the part of the exochiton remaining above it. The oocyte then remained in an inverted position, attached to the exochiton by the apical collar of mesochiton which then began to elongate, forming a hollow, cylindrical stalk, carrying the oocyte towards the ostiole (Fig. 4, E). No further development took place in the potash, but, by applying slight pressure to the coverglass, the oocyte, surrounded only by the endochiton, could be made to pass through the mesochiton, and to remain attached to it in a distal position.

In other cases, when the oogonia were placed in potash, the mesochiton immediately began to extend, eventually rupturing the exochiton below its

region of attachment. The collar then bulged through the split and straightened out (Fig. 4, h), carrying the oocyte towards the ostiole. In many other cases the apical collar rapidly extended in the potash, crumpling under the pressure, but was unable to rupture the exochiton, possibly because the oogonia were less mature.

In both these patterns of extrusion the final result is the same, in that the oocyte, in an inverted position, remains attached to the exochiton by a stalk formed from the apical collar, the main difference being an inversion in the order of the actual extrusion and the extension of the collar to form the stalk.

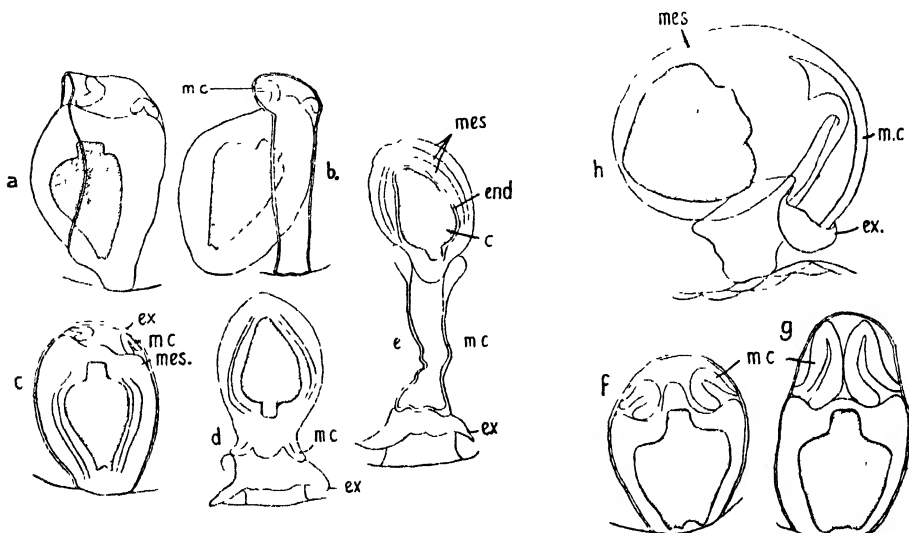


FIG. 4. Stages in the extrusion of the oocyte obtained by treatment with potash. *a-e*, Oogonia in which the elongation of the mesochiton stalk (*m.c.*) took place *after* extrusion. *f, g, h*, Oogonium in which the mesochiton collar became elongated *before* extrusion (*f* and *g*), forcing back a cap of exochiton to which it became attached. Lettering as in Fig. 3.

Without observation of the living plant it is not possible to say which of these methods occurs under natural conditions. The second type offers a more satisfactory explanation of the rupture of the exochiton, but the first method reproduces more closely the stages seen in the untreated sections.

Neither of these methods of escape offers any explanation of the presence of a few empty exochitons, to which mucilaginous strands were attached, not to the turned-back cap of exochiton as seen in these two methods, but to the main part of the exochiton. This appearance could only be achieved if the exochiton were ruptured above the attachment of the apical collar, and not below it, as in all the cases observed. If this were the case, then the oocyte could only escape by passing through the collar or stalk in a manner similar to that described for *M. Urvilliana* (Delf, 1937).

In none of the cases observed was the stalk sufficiently long to carry the oocyte out through the ostiole. Further extension may possibly occur in a manner similar to that seen when pressure was applied to the coverglass

(p. 144). The significance of the three layers of the mesochiton was not seen in these experiments, but they may possibly be concerned with the still further elongation of the stalk, the oocyte passing through the layers one at a time, instead of passing through the mesochiton as a whole. Dawson (1941) figures layers in the mesochiton of *Cystoseira foeniculacea* (Fig. 2, E) and describes the fully extended stalk as being composed of several segments, of which the 'three or four segments correspond to the original layers of mucilage laid down, whose proportions change from flattened to elongated cylinders'. This seems to be a device for the elongation of the stalk similar to that suggested for *S. dorycarpa*.

The inversion of the oocyte, so clearly seen in *S. dorycarpa* on account of the shape of the oocyte, has not been described elsewhere. In *M. Urvilliana* the oocyte did not retain its shape after extrusion, so its orientation could not be seen, but it was suspected that it was in an inverted position. Inversion of the oocyte is postulated, though not actually observed, by Blomquist (1945) for *Turbinaria turbinata*. From a study of the figures it appears that inversion during extrusion also occurs in *Bifurcaria brassiciformis* (Delf, 1935), in *Carpophyllum flexuosum* (Dawson, 1940), and in *Cystoseira foeniculacea* (Dawson, 1941).

The stalk formed in *S. dorycarpa* very closely resembles those of *M. Urvilliana* and *M. boryana*, in both of which there is a similar formation of hollow cylindrical stalks by the extension of a folded, apical collar of mesochiton.

Attaching stalks also occur in species of *Sargassum* (Tahara, 1913; Delf, 1935), *Cystoseira* (Dawson, 1941), *Carpophyllum* (Dawson, 1940), *Bifurcaria* (Delf, 1935; Laing, 1941), and in *Turbinaria* (Blomquist, 1945); but in all these cases the stalks are *solid* and formed by the extension of an apical *pad* of mesochiton.

DISCUSSION

The wall of the oogonium always seems to play a part in the liberation of the oospheres of the Fucales. Its active part does not always end, as in *Fucus*, with the liberation of the oospheres into the cavity of the conceptacle, but in many cases the mesochiton is organized for carrying the oospheres through the ostiole, and is responsible for anchoring them to the parent plant until after fertilization has been achieved, and in some cases even until the sporelings have been established.

All the species in which the formation of such mesochiton stalks has been recorded live in permanently, or almost permanently, submerged habitats. Consequently the retention of the oosphere on the parent plant is a feature of importance with regard to fertilization, as the chances of fertilization must be much greater than if the oosphere were liberated free into the sea, where it would soon sink beyond the range of the spermatozooids which remain nearer the surface while active.

In some species of *Sargassum*, in *Cystophyllum*, and in *Cystoseira* (Dawson, 1941), the stalks also assist in the establishment of the sporelings, before

they are liberated by the dissolution of the mucilage. The material of *S. dorycarpa* was not sufficiently mature to determine if this was the case here also.

With the exception of *Bifurcaria laevigata*, where four oospheres and four short and rather transient stalks are produced by each oogonium, the formation of mesochiton stalks is only reported in species producing only one oosphere in each oogonium.

Another feature which appears to be correlated with the retention of the oospheres is their late maturation. The extrusion of multinucleate oocytes is recorded in *Sargassum Horneri* (Tahara, 1913), in *S. linifolium* (Nienburg, 1910), in *Coccophora Langsdorffii* (Tahara, 1929),¹ in *Marginariella Urvilliana* (Delf, 1937), and in *Turbinaria turbinata* (Blomquist, 1945). In *Cystoseira osmundacea* (Gardner, 1910)¹ and in *C. barbata* (Nienburg, 1910), where there is no retention of the oosphere, the maturation is completed before liberation, but in *C. foemulacea* (Dawson, 1941), where stalks are formed, the oocyte is liberated in a multinucleate condition. This late maturation is carried to a still further extent in *Sargassum Horneri* and *Cystophyllum sisymbrioides* (Tahara and Shimotomai, 1926), where eight equally viable nuclei are present until one of them is fertilized in the presence of the remaining seven.

The formation of multinucleate oospheres can be induced artificially in *Fucus* if the plants are kept at temperatures from 25–26° C. (Whitaker, 1931).² These 'giant eggs' seem to bear a certain resemblance to the multinucleate oocytes of these stalk-forming types. This is of interest, since the members of the Fucales which extrude multinucleate oocytes are generally inhabitants of warmer waters than the intertidal forms which do not exhibit this feature.

Stalk formation occurs in both the Fucaceae and in the Sargassaceae, but is more common in the latter group, where it occurs in several species of *Sargassum*, *Cystoseira*, *Cystophyllum*, *Carpophyllum*, *Bifurcaria*, and *Turbinaria*. Amongst the Fucaceae it is only recorded in two species of *Marginariella*, in *S. dorycarpa*, and possibly also in *Seirococcus* and *Phyllospora comosa*.

The attaching stalks are of two kinds, either solid or hollow (cf. p. 146). Dawson (1941) suggests that this difference in organization may be of systematic value, and points out that it supports the division of the Fucales into the two major groups of the Fucaceae and the Sargassaceae, the former characterized by the possession of hollow stalks, the latter by solid stalks. The few types described since the publication of Dawson's paper also support this view. *Turbinaria turbinata* (Blomquist, 1945), a member of the Sargassaceae, possesses solid stalks, whilst *S. dorycarpa*, one of the Fucaceae, has hollow stalks.

The position of *Bifurcaria laevigata* is interesting in this respect. This plant shows affinities with both the Fucaceae and the Sargassaceae, but is perhaps

¹ Cited from Dawson, 1940.

² In material of *Fucus spiralis*, after the release of the individual oospheres from the endochiton into the sea-water, I have seen up to eight of these oospheres coalesce quickly and easily, forming multinucleate 'giant eggs'. This demonstrates the ease with which this fusion can take place, even after the escape of the oospheres and their release from any pressure exerted by the oogonial wall.

more closely allied to the Fucaceae (Laing, 1941). Each of its four oospheres forms a short *solid* attaching stalk similar to those typical of the Sargassaceae. Thus in the matter of stalk formation, *B. laevigata* more closely resembles the Sargassaceae than the Fucaceae.

Whether they are members of the Fucaceae or the Sargassaceae, the stalk forming fucoids have the following features in common:

1. The stalk is formed from the mesochiton.
2. They are inhabitants of deep water.
3. They have only one oosphere per oogonium (except *B. laevigata*).
4. The maturation of the oosphere takes place at a late stage.

The stalk forming members of the Fucaceae also have the following features in common, viz.:

1. They are specialized and endemic genera.
2. They are all types in which the receptacles are specialized lateral branches.
3. The stalks produced are hollow, contrasting with the solid stalks of the Sargassaceae.

SUMMARY

The thallus is made up of three tissues, meristoderm, cortex, and medulla, resembling those of Fucus.

The receptacles are specialized lateral branches which arise in linear series from the margins of the axis in longitudinal grooves. Their internal structure is similar to that of the rest of the axis. Both male and female conceptacles occur on the same receptacle, the latter in greater abundance. Hermaphrodite conceptacles also occur.

The oocyte has a characteristic amphora shape. At maturity the oogonial wall is very swollen, and consists of three principal regions, the exochiton, mesochiton, and the endochiton. The mesochiton is composed of two parts—an apical collar, ear-shaped in optical section and of a tougher consistency than the rest of the mesochiton, which consists of a very swollen portion completely surrounding the protoplast and is itself differentiated into three concentric lamellae.

During the extrusion of the oocyte the apical collar of the mesochiton extends to form a tubular stalk which attaches the extruded oocyte, in an inverted position, to the empty exochiton. This extension may take place either before or after the rupture of the exochiton.

The structure of the oogonial wall of *Scytothalia dorycarpa* resembles that of Marginariella, another member of the Fucaceae, which also forms hollow attaching stalks. These stalks differ in organization from the attaching stalks found in a number of the Sargassaceae, where they are solid throughout. This difference in the organization of the attaching stalks may be of systematic value.

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A Jurassic Member of the Araucariaceae

BY

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With four Figures in the Text

INTRODUCTION

FOSSILS attributed to the Araucariaceae are known from early Mesozoic times onwards, but they only become really common in Middle Jurassic strata, where they are of very wide distribution. These fossils belong to the following categories:

1. Secondary woods, as petrifications.
2. Leafy shoots, as compressions and as casts.
3. Seed-bearing cone-scales, as compressions and as casts.
4. Cones, as petrifications.

In practically all cases the fossils are entirely isolated. The petrified cones, when fully studied by means of sections, undoubtedly provide good evidence of the nature of the specimen. The evidence provided by the other categories of isolated organs that the plant bearing it was a true member of the Araucariaceae, though it may be strong, is never wholly convincing.

The present communication is concerned with Araucarian remains from the mid-Jurassic Estuarine Series of north Yorkshire, and it is of interest in providing evidence from the shoots and cone-scales of the same species; the male cones are of special interest as the first examples in the fossil Araucariaceae.

The material includes:

1. The main axis and small leafy twigs; form and cuticle structure.
2. The female cone-scales; form, cuticle, and part of internal structure.
3. The male cone; general form, cuticle, and pollen.

Important features remaining unknown are:

1. Internal structure of leafy shoots and of secondary wood.
2. The form of the whole female cone and, of course, all details of gametophytes and other soft parts.

EVIDENCE FOR ASSEMBLING THE DIFFERENT ORGANS

In this paper I have described the vegetative shoots, then the female cone-scales, followed by the male cones. Finally, each in turn is compared with *Araucaria*.

The male cones are found still attached to typical leafy shoots, so that there is no doubt at all about them. The female cone-scales, however, are all isolated, and they are attributed to the shoots on the evidence of association and agreement in structure.

(a) *Evidence of association.* (I refer only to specimens where localization evidence seems to me reliable.)

The shoot *Brachyphyllum mamillare* is a very common fossil and the cone-scale *Araucarites phillipsi* a frequent one in the Estuarine Series of north Yorkshire, and they are found associated. At the time of writing, 125 localities yielding determinable plants have been discovered. *B. mamillare* has been found in 18 as ordinary fossils. (It has been found as cuticle fragments in macerations of coals from a further 20, but as the cone-scale is structurally unsuited to be recognized after this treatment, these cuticle localities may be omitted.) Of these 18, no less than 13 yield *A. phillipsi*, usually in the same bedding plane and on the same slab of rock as numerous shoots. In some of these localities (e.g. Biggersdale Slack, Sandsend) the association is most impressive, as there is no other species at all present. In others it is only slightly less impressive, there merely being a number of ferns, and perhaps Bennettitales in addition. In a few (Gristhorpe), where there are in addition many unassigned conifer shoots, the association means very little. (A list of these localities is included in a thesis deposited in the Library of Reading University.)

It is doubtful if *A. phillipsi* ever occurs without the shoot. It has in fact been recorded without the shoot three times, but all are very inconclusive. Two refer to pieces of shale fallen from the cliffs south of Whitby where nothing further is known of the associates than is presented on the face of these pieces of rock, and one refers to a specimen collected by Mr. Wonnacott from the (?) Upper Estuarine Cliff at Gristhorpe, without record of associates.

Although the evidence of association has often been impugned, I think that anyone who, from locality after locality, had collected slabs of shale showing *B. mamillare* shoots and *A. phillipsi* cone-scales, could not fail to be impressed.

A species of *Araucarites* was collected by M. Black from the Upper Estuarine of North Bay, Scarborough, without *B. mamillare*, but with many shoots of another species of *Brachyphyllum*, namely, *B. scalbiensis*. The cone-scales are all preserved, and although I suspect that they may be distinguishable from *A. phillipsi*, I prefer to leave them undetermined.

(b) *Agreement in structure*

The agreement in structure of the cuticle of the leaf of *B. mamillare* and the tip of *A. phillipsi* is perfect in detail, the only difference being that the tip of the cone-scale is more pointed. This type of cuticle happens to differ from that of every single known leaf in the Yorkshire flora, and not only the

described species, but the much more numerous undescribed leaf fragments occurring in the coals have been compared.

DESCRIPTION

Vegetative parts

The description of the vegetative parts will be brief, as I have described them elsewhere (Kendall, 1947).

The usual type of shoot resembles that shown in Fig. 4 (apart from the male cones), a stem bearing weak laterals in a more or less pinnate manner, and the whole covered with bulging leaves. The leaf is minute, virtually consisting of an extremely swollen leaf-base cushion, rising to an obtuse edge which represents the leaf-apex. The diagram given (Fig. 2 c) shows what I believe to have been its original form. Unfortunately, there is no evidence at all of the number of the veins in the leaf.

There is evidence that much stronger stems, possibly representing the main axis or leader, exist. Such specimens are rare, and are stout, with many ranks of large leaves (Fig. 2 E and F). The identity of such specimens is shown by their agreement in cuticle. No specimen yet collected shows the branching of these large specimens.

The cuticle (Fig. 2 B) is moderately thick, and shows plenty of stomata on the exposed surface of the leaf-base cushion, but only a few on the adaxial surface. In fundamental stomatal structure it agrees with all conifers; it also shows features of generic agreement with *Araucaria*, for instance:

1. The rounded stomatal apparatus, with numerous encircling cells.
2. The tendency of the stomata to form longitudinal rows, but to have variable orientation in their rows.
3. The fact that the stomatal rows are not grouped into bands. Among the specific characters are the rounded epidermal cells, each with a large thickened area on the outer wall.

Comparison

B. mamillare shoots look rather like *Cupressus* in their pinnate branching and minute leaves, but differ from all Cupressaceae in their spirally arranged leaves. The only conifers showing real agreement in shoot form are certain Podocarpaceae, certain Taxodiaceae, and certain species of the Araucariaceae, where, however, the leaves are larger. In cuticle, however, it agrees with the Araucariaceae, but differs more or less seriously from the other two. For instance, the Podocarpaceae of similar shoot form have longitudinal stomata, and the Taxodiaceae rather different encircling and subsidiary cells.

Other Jurassic conifers provide a series of species with similar cuticles, ranging from *B. desnoyersii*, with even more reduced leaves than *B. mamillare*, and *B. scalbiensis*, with larger leaves than *B. mamillare*, to various species of *Pagiophyllum* with still larger leaves, some of them as well developed as in recent species of *Araucaria* and looking very like them. In view of the fact that all these species are less well known than *B. mamillare*, and their own

classification therefore insecure, the support given is not fully reliable, and all that can be said is that it is reasonable to classify *B. mamillare* shoots, on the evidence that they themselves provide, in the Araucariaceae, at least provisionally, and to regard *B. mamillare* as a reduced ally of, say, *Araucaria excelsa*.

THE FEMALE CONE-SCALE *ARAUCARITES PHILLIPSI* CARRUTHERS

Type-specimen 305 in the Leckenby Collection, Cambridge.

This cone-scale has not been described since 1900, when little was known about it, and it is, therefore, described here in some detail.

References

1829. *Cycadites*, Phillips, p. 150.

1829. Winged seed, Phillips, p. 190, pl. x, fig. 5.

1869. *Araucarites phillipsii*, Carruthers, p. 6, pl. 11.

1900. *Araucarites phillipsi*, Seward, p. 285, pl. x, fig. 4.

Seward (1900) gives a few other references to citations of this species in the literature.

Description

Female cone-scales resembling the present material were recorded by Phillips (1829) from the Middle Jurassic of Yorkshire (Lower Estuarine of Haiburn Wyke). These he first took to be the seeds of *Cycadites* (p. 150), but later (p. 290) he referred to them simply as a 'winged seed'. Carruthers (1869) referred them to *Araucarites*, and also identified them with a badly preserved specimen (from the same deposit) which he took to be a complete cone. However, I think it more likely that this specimen was actually a Cycad shoot covered with leaf-scars, and in any case I know of no reason for identifying it with *Araucarites phillipsi*.

The present material consists of numerous isolated female cone-scales from the Estuarine Series of Yorkshire. In gross form, and as far as can be determined, in minute structure also, they appear to form a fairly uniform group.

The scale is always cuneiform, but rather varied in size and shape; the figures (Fig. 1 A-J) give the range. That shown in Fig. 1 A and B is about the commonest form. Double scales, like those shown in Fig. 1 C and D, are rare. Thus variation, although great, is paralleled by what is seen in a single recent *Araucaria* cone.

The pointed apex has usually been flattened as a result of compression, but occasionally still points upwards at right angles to the scale (V. 23962). The lateral margins of the scale are membranous, and the middle part not very thick, usually about 200μ , which is the thickness of a fairly stout leathery leaf in this flora.

A ligule (Fig. 1 B) is present, about 6 mm. broad, and quite short. The form of its apex is unknown because it had broken off in all the specimens which I studied. Its substance is very thin and fragile in the fossil state.

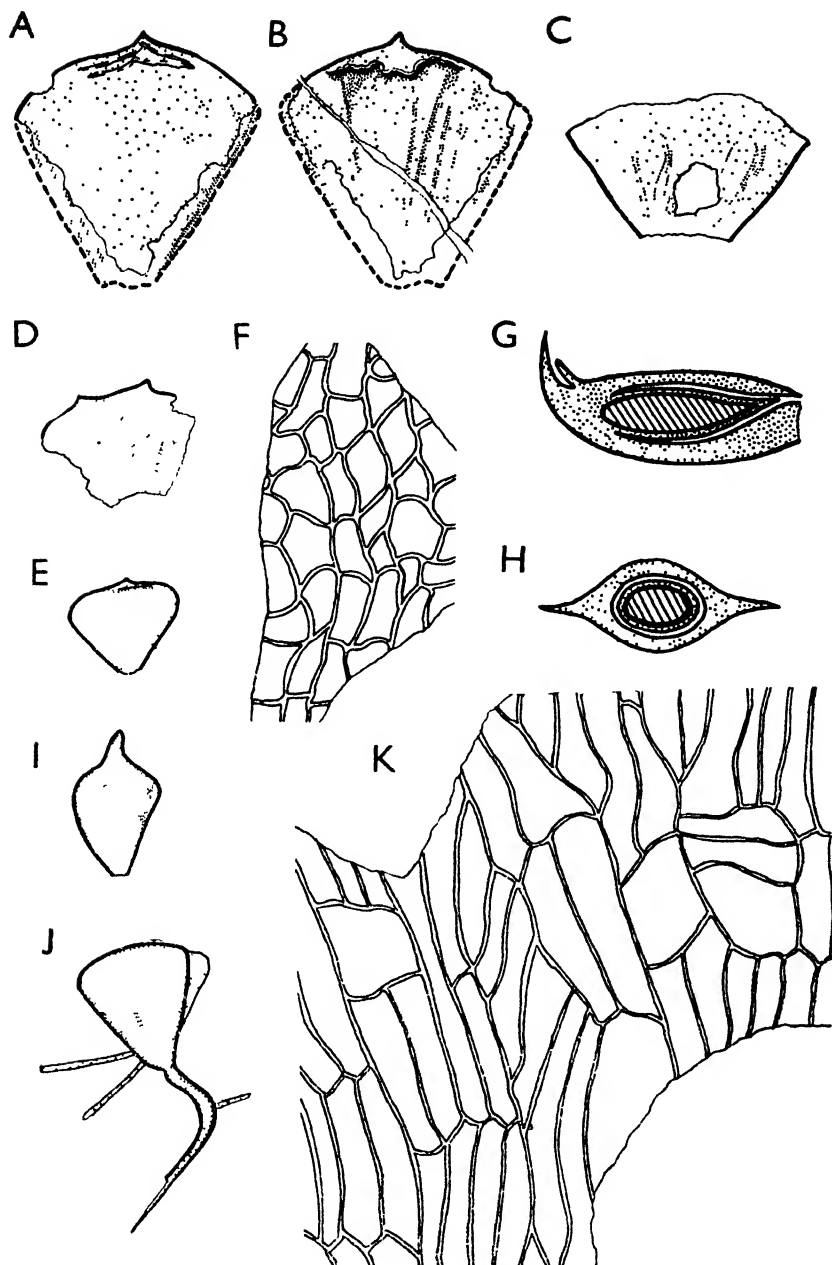


FIG. 1. *Araucarites phillipsi*. A, *A. phillipsi*, lower side, edge of coaly substance shown by thin black line, edge of cast in matrix by broken line. Double lines near apex represent folds. (V. 27551, $\times 2$.) B, Transfer preparation of specimen shown in A, showing base of ligule (unstippled area represents crack in substance). ($\times 2$.) C, *A. phillipsi*, double-seeded scale. Nodule of pyrites in centre shown white. (V. 27600, $\times 1$.) D, *A. phillipsi*, double-seeded scale, with two pointed tips. (V. 27563, $\times 1$.) E, *A. phillipsi*. (V. 27546, $\times 1$.) F, Cuticle of ligule. (V. 27560, $\times 200$.) G, Reconstructed longitudinal section of unaltered cone-scale, endosperm shaded, substance of nucellus and scale stippled. ($\times 2$.) H, Reconstructed transverse section of unaltered cone-scale, shading as in G. ($\times 2$.) I, *A. phillipsi*. (V. 27575, $\times 1$.) J, Seedling, Oxford Museum (J. 5007, $\times 1$.) The lateral root-like bodies may not be continuous with the cone-scale. K, Cuticle of outside of cone-scale. (V. 27574, $\times 200$.)

Transfer preparations of good specimens show that on both sides the surface of the scale is continuous, and not folded, and the seed was certainly buried in the substance.

Cutinized membranes. The following membranes are cutinized:

1. The outside of the scale, continuing into the pointed apex and the ligule. (As the seed is embedded, this also forms the outside of the integument.)
2. A fairly robust membrane present only at the proximal end of the cone-scale, and here regarded as belonging to the epidermis of the nucellus.
3. A rather unevenly thickened membrane, regarded as occurring in the substance of the nucellus.
4. The megaspore membrane.

1. *Outside of scale.* The cuticle of the main part of the scale (Fig. 1 K) is a firm membrane, marked by the walls of cells which are $20-40\mu$ wide, but rather variable in length; the average is perhaps 120μ long, but cells may reach 200μ long. The shortest are at the distal end of the scale, where they are approximately square. The cells are arranged in groups of sister-cells, as shown in the figure; these groups may be curved. No stomata were seen in the fragments observed, and I believe that none occur here. The cuticle of the membranous edges is rather evenly thickened, so that the walls of the epidermal cells are obscurely marked. The markings suggest that the cells were short.

The cuticle of the pointed apex (Fig. 2 A) is firm, and is indistinguishable in detail from that of *Brachyphyllum mamillare* (Fig. 2 B). The cell-walls are marked by ridges about 10μ wide over most of the point, but these become narrow near the base, where the cuticle continues into that of the main part of the scale.

The epidermal cells of the ligule (Fig. 1 F) are distinctly marked, rectangular, and $40-60\mu$ long by about 20μ broad. Stomata are absent.

2. *The cuticle of the nucellus.* This cuticle (Fig. 2 D) is not very extensive; it is firm, and is marked by elongated cells with minutely crenulated walls. These cells, which are arranged in groups of sister-cells, are $100-120\mu$ long by $12-20\mu$ broad. The groups are often strongly curved, but are never transversely orientated.

3. *Unevenly thickened membrane.* This membrane is not figured, as it does not show any clearly defined markings. It is extensive, occurring in preparations from all parts of the seed. In some specimens at least, it is thickest at the proximal end of the scale, and in some specimens it gives the impression of being more than one cell deep. Rather obscure longitudinal markings are present.

4. *The megaspore membrane.* The megaspore membrane is not figured because the markings on its surface are obscure. It is firm, and deeply staining, but not granular. In many specimens it is flat, but in others it has been thrown into folds, probably owing to the shrinkage of the endosperm prior to fossilization. Faint longitudinal striations are sometimes present.

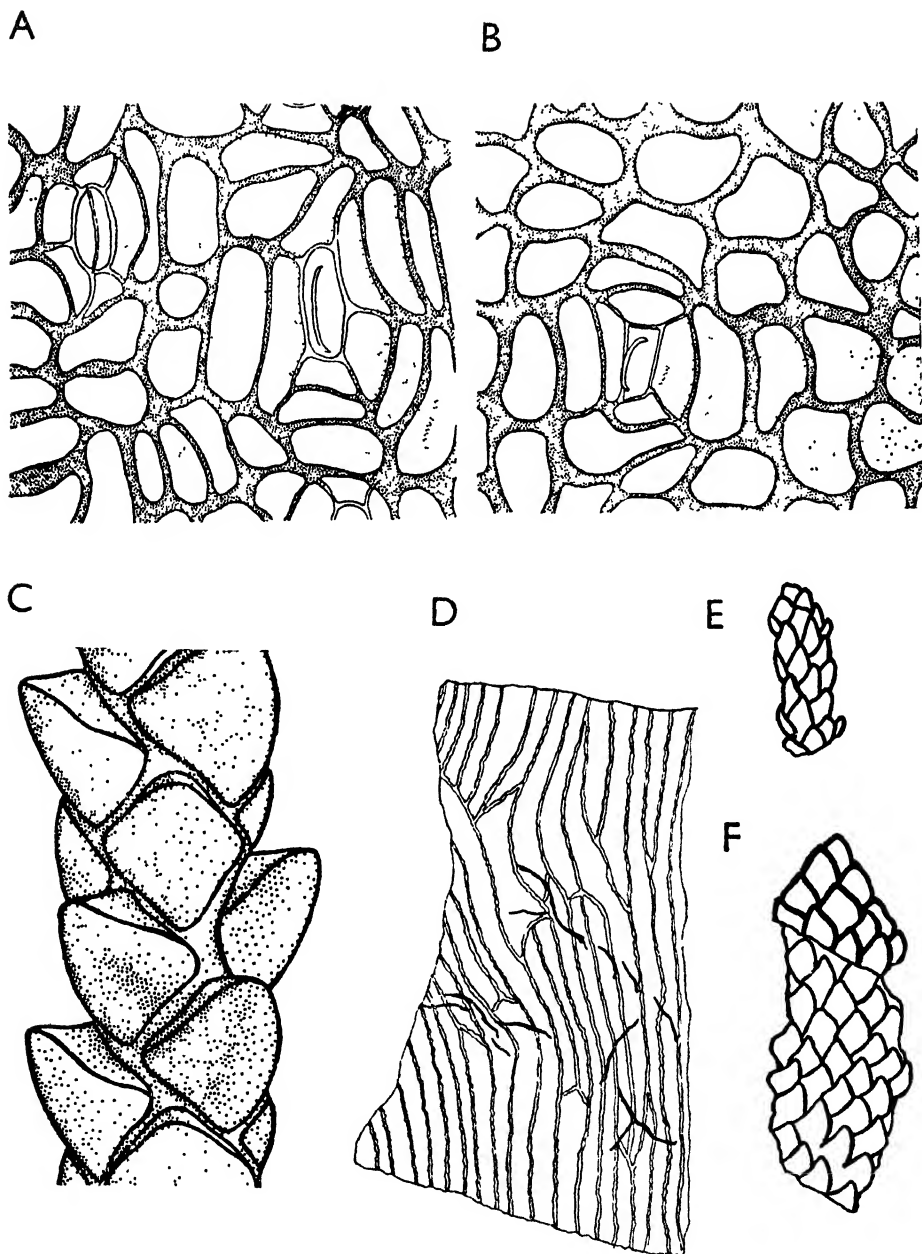


FIG. 2. *Brachyphyllum mamillare* and *Araucarites phillipsi*. A, *A. phillipsi*, cuticle of pointed apex, antichinal walls of epidermal cells closely stippled, thickened areas of outer walls lightly stippled. (V. 27560, $\times 400$.) B, *B. mamillare*, stippling as in A. ($\times 400$.) C, Restoration of unaltered *B. mamillare* twig. ($\times 5$.) D, *A. phillipsi*, cuticle of epidermis of nucellus, walls of cells represented by thin, double lines, folds in membrane shown by thick, black lines. (V. 27574, $\times 200$.) E, *B. mamillare*, main axis. (V. 27607, $\times 1$.) F, *B. mamillare*, main axis, substance shown by thick, cast in matrix by thin, lines. (V. 27615, $\times 1$.)

Discussion, and comparison with cone-scale of Araucaria

This interpretation, while it is not the only possible one, is put forward because it appears to account for the features found in *A. phillipsi*.

Practical difficulties were great, and so mistakes of fact are possible. During maceration the fossil breaks into minute fragments, rarely exceeding 0.5 mm. broad. Each fragment (from the region of the seed) consists of a pile of membranes, separated by layers of coaly material, and since the membranes are easily dissociated and disrupted by slight movements of the ammonia during the last stage of maceration, it is difficult to determine their number and their sequence. The outer cuticle of the scale is robust and easily recognized, but the inner cuticles are more delicate, as well as variable in appearance, and the full complement is not always present. Moreover, the membranes tend to remain together in pairs (forming apparently combined membranes), although they have never adhered during fossilization.

There is also a difficulty of interpretation about the nature of the unevenly thickened membrane (3). This is thought to have been recognized in the recent *Araucaria araucana*, but here it differs in being far less extensive. Its position in the recent scale is in the substance of the nucellus, near the micropyle, but its true nature is not understood.

General Comparison

The external form of the cone-scale with its free tip, its ligule, and its embedded seed agrees perfectly with *Araucaria*. So, too, does the occasional presence of two seeds. There is remarkably close agreement between the cuticle of the outside of the cone-scale in *Araucarites phillipsi* and *Araucaria araucana*. The nucellus is believed to be free in its upper part at least in *Araucarites phillipsi*, and it may be free more extensively, but this is unproved. In the recent genus it is free to the base, but it is noteworthy that it is its apical part that is cutinized and so recognized by the methods used here for the fossil.

Araucarites phillipsi is of the broad type, with membranous wings, to be seen in the Section Eutacta, rather than the cylindrical type of the Section Colymbea. In no respect does *A. phillipsi* resemble the cone-scale of *Agathis* rather than *Araucaria*. It differs still more from the cone-scale of any other genus.

Seedling

The specimen shown in Fig. 1 j is a normal scale of *A. phillipsi* which appears to have germinated. What looks like a stout radicle protrudes from the base, and although the radicle is reduced in the fossil to a delicate brown film, it does appear under the microscope to be continuous with the scale. Higher up there are some slender structures which are represented in the drawing, although I think these are most likely to be plant fragments fortuitously lying under the scale. From their position it is unlikely that they represent branch roots.

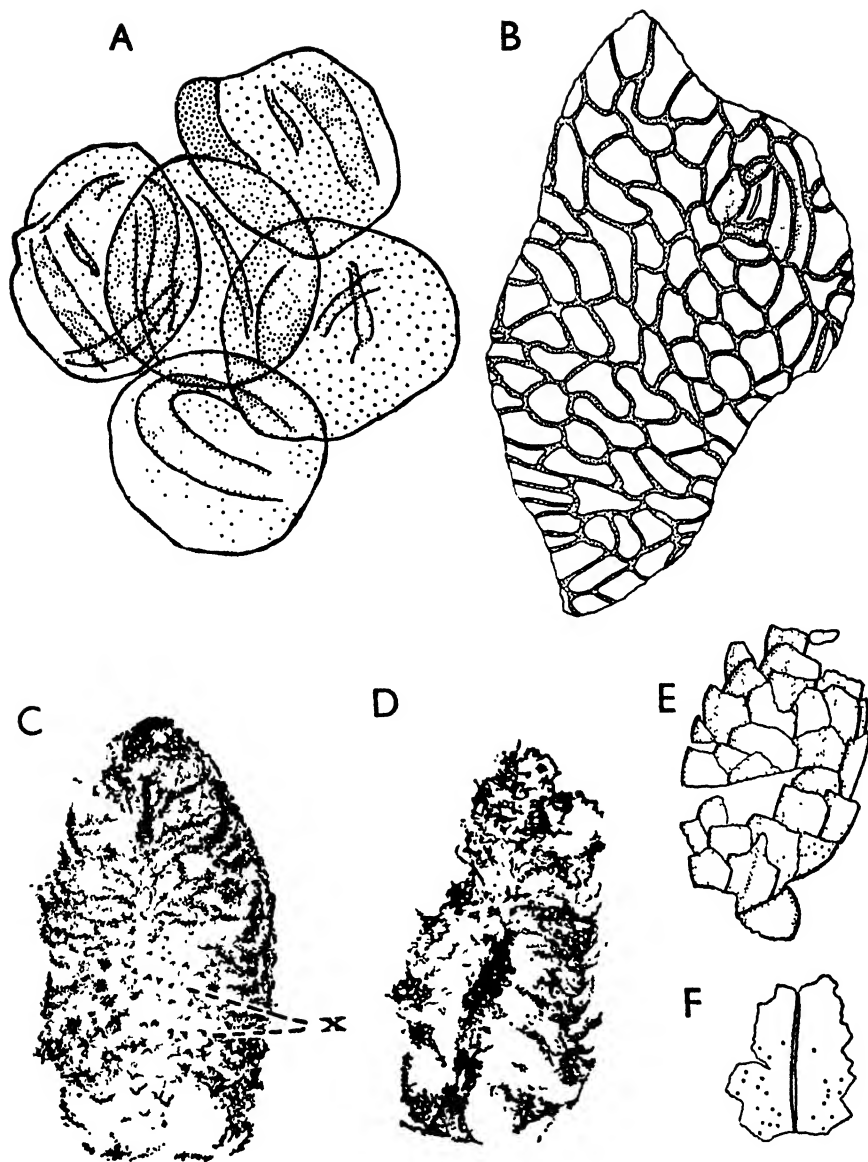


FIG. 3. *B. mamillare* and male cones, and *A. phillipsi*. A, Group of pollen grains. (V. 27554, $\times 400$.) B, *B. mamillare*, male cone, cuticle of tip of microsporophyll. (V. 27548, $\times 400$.) C, *B. mamillare*, longitudinal section of male cone embedded in Bakelite resin, cone axis showing at tip, sections of sporophyll stalks showing at x. (V. 27579a, $\times 5$.) D, Longitudinal section through cone-axis of cone shown in C. ($\times 5$.) E, *B. mamillare*, male cone, showing broken tips of microsporophylls. (V. 27548, $\times 4$.) F, *A. phillipsi*, cuticle of pointed apex, each dot represents one stoma. (V. 27575, $\times 10$.)

Fossil seedlings are rare, and (assuming its interpretation) this specimen is unique. As it happens, the seedling is important in the classification of *Araucaria*, for in the Section Eutacta (including *A. excelsa*) the cotyledons are epigeal, while in the Section Colymbea (including *A. araucana*) hypogeal.

Unfortunately this seedling is rather young, but, even so, the stout radicle does rather suggest epigeal germination.

THE MALE CONE OF B. MAMILLARE

Description

The male cones attached to the shoot shown in Fig. 4 are possibly immature, but all the others are post-mature, with nearly empty pollen-sacs.



FIG. 4. *Brachyphyllum mamillare*. *B. mamillare* shoot, with male cones, Oxford Museum (J. 933, $\times 1$).

The figured specimen (which was borrowed) could not be investigated by chemical means, but nearly all the others were so investigated. Many are preserved as a thin, flat film of coal; transfers of these show the free ends of the microsporophylls and little else. A few cones are more solid, there being a certain amount of rock matrix between the sporophylls. These more solid ones were impregnated with Bakelite resin (to make the matrix more coherent)

and the surface was rubbed away to expose a sectional view. These sections (Fig. 3 C and D) disclosed the cone-axis and the long stalks of the sporophylls, but the tissues are represented by mere specks of coal, no true petrification having occurred.

Maceration of the sporophylls yields cuticles which are well developed in the free part of the sporophyll, but elsewhere very thin, especially in the pollen-sac. The cuticle of the free part of the sporophyll (Fig. 3 B) resembles that of the leaf of *B. mamillare* (Fig. 2 B) in the general characters of both its epidermal cells and its rather numerous stomata. The cells, however, do not form well-defined rows, and the encircling cells of the stomata are rather irregular.

Pollen grains (Fig. 3 A) are obtainable in small numbers at least, from every cone macerated. They are small, round, and 60–80 μ in diameter. The wall has been thrown into a series of irregular folds on collapsing. Pits and granules are absent from the extine.

It is unfortunate that no details can be supplied about the pollen-sacs. In the ripe cones their walls are delicate wisps of blackish matter detectable only with difficulty among the sand grains in the sections. It is not possible to say whether the sacs are definitely free from the stalk, though no sign of attachment was noticed.

The male cone of *B. mamillare* is of smaller size than in many (or perhaps all) of the modern Araucarians, but agrees in its terminal position on the shoot. It agrees also in the long stalk and the upturned end of the sporophyll, and in the round, wingless pollen grain. It is not possible to say whether it agrees in its pollen sacs with the Araucariaceae.

SUMMARY

1. The vegetative shoots of *Brachyphyllum mamillare* are briefly described.
2. The female cone-scale *Araucarites phillipsi* is described in detail. It is shown that it has seed with a free nucellus.
3. The male cone and pollen are described.
4. Evidence of association and agreement in structure is adduced for referring *Araucarites phillipsi* to the same plant as *Brachyphyllum mamillare*.
5. Comparison with *Araucaria* shows agreement on very numerous points between these various organs, but several gaps in knowledge are pointed out.

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Experimental and Analytical Studies of Pteridophytes

XIV. Leaf Formation and Phyllotaxis in *Dryopteris aristata* Druce

BY

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With Plates III, IV, and V, and forty-four Figures in the Text

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1. INTRODUCTION

THE term *phyllotaxis* refers to the arrangement of leaves upon the axis or shoot and to the underlying geometrical principles. Of the many accounts of phyllotaxis only a few have been based on experimental work, these relating to hypotheses which are mechanical in conception. Other hypotheses, in which the arrangement of leaves is attributed to some unknown inner property of the shoot apex, are not suitable in their present form for experimental treatment. The principal experimental investigations—those of M. and R. Snow—as well as a majority of the other relevant researches, relate to flowering plants. Since the ferns show leaf arrangements comparable with those found in flowering plants it seemed desirable that the experimental work of previous investigators should be repeated and extended using fern apices, the more so as in their organization these apices show a number of characteristic and distinctive features.

In this paper both leaf formation and phyllotaxis in ferns are considered. The general aim has been to advance our knowledge of morphogenetic processes at the apical meristem. Particular aims were: (i) to test experimentally

two hypotheses of previous investigators, (a) that the position to be occupied by a leaf primordium is determined by an acropetally developing pre-existing leaf-trace, and (b) that the next primordium to be formed arises in the first space of sufficient width above and between existing primordia of the top whorl; (ii) to investigate phyllotaxis and leaf formation by other experimental methods; and (iii) to reconsider the anatomy of leaf formation in ferns. In the past, investigators of phyllotaxis from a mathematical standpoint have paid insufficient attention to the relevant physiological aspects; those whose interest has centred on leaf formation have tended to neglect the positional aspect. It seems desirable that the two aspects should be examined together. Elsewhere (Wardlaw, 1949), observations on leaf formation in *Dryopteris* have been illustrated and described in some detail; these data are referred to summarily in the present paper.

2. RÉSUMÉ OF EARLIER INVESTIGATIONS

No attempt will be made here to deal comprehensively with the general literature of phyllotaxis. Summaries of earlier investigations are to be found in standard texts and in the writings of Church (1904), van Iterson (1907), M. and R. Snow (1931, 1933, 1935, 1947, 1948), Priestley and Scott (1933), Priestley, Scott, and Mattinson (1937), Sifton (1944), &c. In two recent papers M. and R. Snow (1947, 1948) have indicated the hypotheses which can be, or have been, put to experimental tests as follows: (1) that the position in which a new primordium will appear is determined by the position of a pre-existing leaf-trace; (2) that the superficial layer of the shoot grows more rapidly than the tissues within and so forms folds which become leaves; (3) that new primordia arise *as far as possible* from the existing primordia, the factors involved being physiological (nutritional) or physical; and (4) that each new primordium arises in *the first available space* on the apex above and between the existing primordia of the top cycle, i.e. in the first space which '*attains both some necessary width and some necessary distance below the extreme growing point*'. They have discussed in some detail experimental data relating to these hypotheses and have concluded that the first three are untenable but that the fourth is substantially supported by the experimental evidence which they have been able to obtain. Ball (1948) has also described experiments which indicate that it is improbable that the position of a new primordium is determined by a pre-existing leaf-trace. Preliminary experiments on the incision of fern apices (Wardlaw, 1947, 1948), comparable with those of M. and R. Snow (1947) on flowering plants, have yielded evidence contrary to the second hypothesis mentioned above.

The fourth hypothesis, that of M. and R. Snow, appears to be compatible with the available experimental evidence. Since it is derived from the earlier researches of Hofmeister (1868) and van Iterson (1907), a brief reference to these works should be made. In his *Allgemeine Morphologie der Gewächse* (1868, p. 508) Hofmeister has advanced an hypothesis in which he suggested: (i) that the outer walls of the superficial cells of the shoot apex offer resistance

to the lateral outgrowth of new organs (primordia); (ii) that this resistance is not uniform over the growing-point, new lateral organs appearing in regions of greatest elasticity; (iii) that in proximity to the last-formed primordia the superficial membrane, being already stretched, has minimal elasticity; (iv) that a new primordium will appear in that position on the apex which lies farthest from the margins of the last-formed primordia, i.e. in the position of maximal elasticity and minimal tension. The mechanical aspect of Hofmeister's theory as outlined above has received little support. That the position in which a new leaf primordium will be formed is affected by the positions of the leaves already present is generally accepted. The new primordium, in Hofmeister's view, arises on the apex in a position which is farthest from the basal edges of the nearest older primordia. In a spiral sequence, for example, the position of the new primordium is held to be not only affected by the position of the last-formed primordium, but by the positions of the several adjacent primordia. But, as M. and R. Snow point out (1931), van Iterson (1907) was able to explain the main facts of phyllotaxis without postulating any tendency for the next leaf to arise *as far as possible* from previous leaves, the fact of observation being that *the new primordia are formed in the largest gaps between the previous ones*. According to van Iterson, the phyllotaxis of any plant depends on the relative sizes of the young leaf primordia and the apex, and the way in which the system began. He developed the hypothesis that the insertion of primordia on the conical shoot apex constitutes a system of touching circles and showed by reference to geometrical data that only certain contact systems are possible. He tested his hypothesis by examining various plants and concluded that, for the species examined, it was correct, though in some species the young leaves are probably not circular in outline.

M. and R. Snow, in a series of ingenious experiments, involving delicate incisions of the minute vegetative shoot apex of different flowering plants, have obtained direct experimental evidence that *each new leaf primordium develops in the first space on the apex that attains a certain minimum width and a certain distance below the extreme tip of the shoot*: 'For the largest gap between the previous leaves will, as a general rule, be the one in which, through the growth of the apex, the minimum space necessary for leaf formation will first become available.' Their data also indicate that each new primordium is determined as a whole in contradistinction to the view of Schoute (1913) that the centre is determined first.

The emphasis, then, is not on the *distance* of the incipient primordium from the last-formed primordia as suggested by Hofmeister and later workers (Schmucker, 1933; Priestley and Scott, 1933)—which M. and R. Snow (1947) describe as the 'repulsion theory'—but on the existence at the apex of *spaces suitable for the development of primordia*—the 'theory of the first available space'. These contrasting views are made clear in the following quotation from M. and R. Snow (1948):

'On the repulsion theory the exact position in which a new leaf will arise must depend on influences exerted by all the existing young leaves in the top cycle round

the apex at the least. But on the theory of the first available space the expectation is different. Around the apex there are a number of depressions or gaps between the leaves of the top cycle, and these gaps become available successively and are occupied by new leaves. So on this theory also the sequence in which the several gaps are occupied by new leaves depends on the positions and shapes at any time of all the existing leaves of the top cycle; and we claim to have shown previously (M. and R. Snow, 1931, 1933, 1935) that this expectation is correct. But the exact position *within* any one of these gaps in which a leaf will be formed depends, in this theory, on the positions and shapes of those leaves only which border the gap, and not on the other leaves of the top cycle.'

Although the ferns have not been neglected as materials for the study of phyllotaxis (Church, 1904), experimental work has so far been confined to flowering plants. From a consideration of organ formation in *Dryopteris aristata* the writer (Wardlaw, 1947, 1947a, 1948, 1949) has arrived at views relating to the mechanics of phyllotaxis which contain elements not found in previous hypotheses. Both on anatomical and experimental grounds the hypothesis has been put forward that during the development of a leaf primordium a tangential tensile stress is induced in the apical meristem above the leaf axil. A further hypothesis is that the next primordium to be formed arises in that region of the apical meristem in which tensile stress is minimal. Studies of the distribution of growth at the apex support these views. Moreover, when apices were laid bare and incised and punctured in various positions, positive evidence was obtained of the existence of a region of tensile stress in and above the axil of each primordium, whereas stress was slight or absent in those regions in which the new primordia would arise. The largest unoccupied space on the apical cone, above the level of the last-formed primordium, is also, in the normal apex, the region in which tensile stress is minimal. It will be seen that in several respects this finding is in close agreement with the views of Hofmeister and M. and R. Snow. While observations such as these may suggest how a particular space on the apical meristem may come to differ physiologically or physically from adjacent areas, they give no direct clue as to why a leaf should be formed in such a position. Hence, in the writer's view, there are, for the time being, two different but interrelated problems for consideration, (i) the factors which relate to *the actual formation* of a leaf primordium; and (ii) the factors which determine *the position* in which leaf formation can take place.

3. THE APICAL MERISTEM IN FERNS

In leptosporangiate ferns the *apical meristem*, as defined by Wardlaw (1943), consists of a superficial layer of prism-shaped cells of distinctive appearance and reaction. These *meristematic cells* are formed by the division of the *single apical cell* (and its segments) and apparently share many of its physiological properties. From an early stage the formation of a leaf primordium can be referred to the division of a single enlarged meristematic cell. In fact, in the literature, the leaf in leptosporangiate ferns has hitherto been regarded as the

product of a single apical cell (Hofmeister, 1868; de Bary, 1884; Bower, 1923; Campbell, 1940; Sifton, 1944; Wardlaw, 1945a). Recent studies have led the writer to the view that this account of the origin of the fern leaf requires revision (Wardlaw, 1949). It may be noted that, at the time of its inception, the primordium lies just within the basiscopic margin of the meristem, i.e. as far away from the apical cell as possible, Text-figs. 1 and 2, and Pl. III, Fig. 1.

In ferns, the region immediately below the apical meristem—the sub-apical region—is characterized by a very considerable enlargement, in the course of which the prism-shaped meristematic cells on the basiscopic margin of the meristem undergo rapid division with the formation of epidermis and cortical parenchyma. The bases of the leaf primordia also show this very extensive growth development, particularly in the tangential direction.

4. METHODS

Apices of stout erect shoots of *Dryopteris aristata* Druce were exposed using methods which have already been described (Wardlaw, 1944, 1947). Incisions were made as required in different positions by means of a small, sharp cataract knife. In removing the dense investment of scales and the older leaf primordia, the primordia round the base of the apical cone are liable to be damaged and occasional injuries to the meristem are almost unavoidable. After treatment the experimental materials were grown in moist peat in a cool greenhouse and kept under observation for several weeks. Photographic records were obtained with the Ultro Pak microscope.

The semi-diagrammatic plan drawings of the apex in the text were obtained by means of the Ultro Pak microscope and camera lucida: these give reasonably accurate records of the positions occupied by primordia, &c. For the detailed inspection of specimens a binocular microscope was used.

Terminology. The terminology of M. and R. Snow (1931) has been followed. The youngest visible primordium is referred to as P_1 in the text and is labelled 1 in the illustrations; the next older primordium is denoted by P_2 , and so on. The position of the next primordium to be formed, but as yet invisible, is denoted by I_1 , and the next after that by I_2 , and so on; these symbols (I_1 , I_2 , I_3 , &c.) are also used to indicate new primordia which have developed during the course of the experiment. The position of I_1 is not difficult to observe: it lies between P_3 and P_5 on an unoccupied flank of the apical cone. With suitable magnification (40 times) and illumination, even a very small protuberance can readily be detected. Emergent bud primordia have never been observed on uninjured, normal apices.

5. EXPERIMENTAL OBSERVATIONS

(a) *Acropetal effect of the leaf-trace*

Sterling (1945) has advanced evidence that in *Sequoia sempervirens* the leaf-traces are formed in advance of the leaves to which they ultimately relate, i.e. an acropetally developing leaf-trace determines the position in which a

new leaf primordium will arise; and Gunckel and Wetmore (1946) consider it probable that in *Ginkgo biloba* the traces are formed before the leaves and determine their position. These suggestions have been subjected to experimental tests by M. and R. Snow (1947, 1948). When the apices of *Lupinus albus* were incised transversely below the presumptive positions of I_1 and I_2 —i.e. primordia as yet quite invisible and not in any way determined—leaf primordia were nevertheless formed after some days. This finding is supported

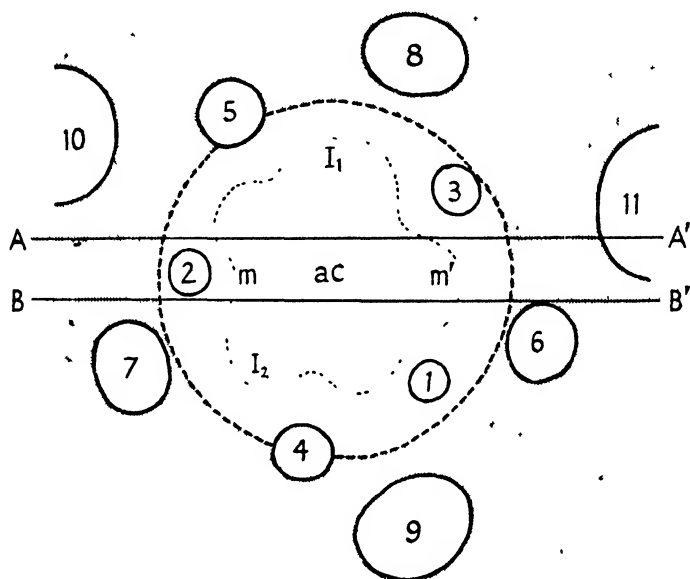


FIG. 1. See opposite.

by the experimental studies of Ball (1948). The experiments reported below are based on the application of the technique of M. and R. Snow to the ferns.

In apices of *Dryopteris aristata*, prepared as already described, small transverse incisions were made below the presumptive leaf positions I_1 , I_2 , and I_3 . The apices were then kept under observation for several weeks. A typical result is illustrated in Text-figs. 3 and 4. Except where the tissue in a locus of leaf formation had been damaged by the incision, a new primordium made its appearance after a few weeks. In other words, the severing of direct connexion with the vascular tissue below did not preclude leaf formation. In other experiments (Wardlaw, 1947, 1949) in which the apical meristem has been isolated by vertical incisions close to the meristem, with complete severing of vascular connexion with the tissues below, new leaf primordia have continued to be formed (see also Section 5 (c)).

(b) *The effect of longitudinal incisions on leaf positions*

If the positions of new primordia are determined by physical factors, e.g. by stresses, or by considerations of space, it might be anticipated that by incising

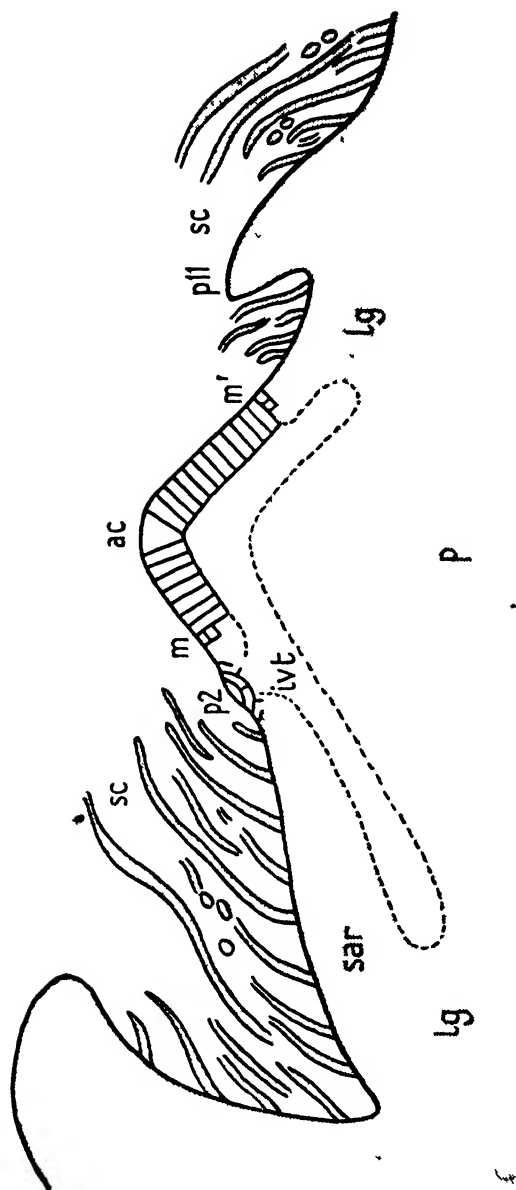


FIG 2

TEXT-FIGS 1, 2 The apex of *Dryopteris aristata* as seen from above and in median longitudinal section in the plane *AA'-BB'*. The circle (broken line, Fig 1) indicates approximately the base of the apical cone, the sub-apical region (*sar*) is stippled, *I*, 2, 3, leaf primordia in order of increasing age, *I*, the position of the next primordium to be formed; and *I*, the position of the next but one to be formed, *m-m'*, the apical meristem; *ac*, apical cell; *πt*, incipient vascular tissue, *lg*, leaf-gap; *p*, pith, *sc*, scales (Semi-diagrammatic $\times 40$.)

the apex longitudinally in various positions the primordia subsequently formed would show some departure from their theoretically normal positions. M. and R. Snow (1931, 1933, 1935, 1947, 1948) have already described in detail interesting experiments of this kind for various flowering plants. They found that if I_1 was isolated the position of I_2 in relation to its adjacent 'contact' leaves was unchanged, but I_3 was always found to be displaced

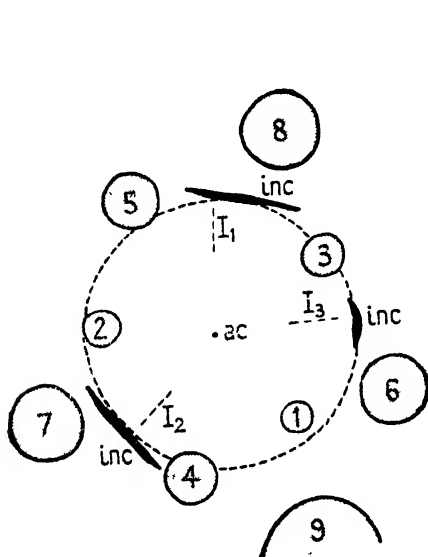


FIG. 3.

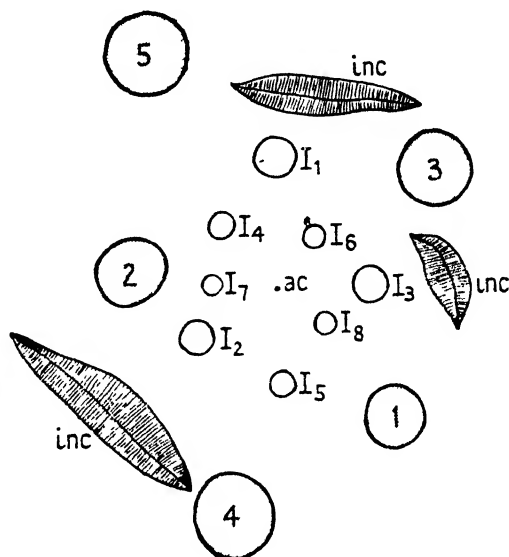


FIG. 4.

TEXT-FIGS. 3, 4. The effect of transverse incisions below the presumptive positions of new leaf primordia.

TEXT-FIG. 3. Transverse incisions (*inc*) of the apex have been made immediately below the positions I_1 , I_2 , and I_3 ; 1, 2, 3, leaf primordia in order of increasing age present at beginning of experiment; *ac*, apical cell.

TEXT-FIG. 4. The same apex later. The three incisions (*inc*) have become distended, but new primordia have been formed in the positions I_1 , I_2 , and I_3 ; in all, eight new primordia (I_1 – I_8) have been formed in normal phyllotactic sequence. (· 15.)

towards the wound. In several experiments they also found that the direction of the phyllotactic spiral was actually reversed.

Before entering on an account of experiments with *Dryopteris aristata*, it seems desirable to place before the reader a clear indication of the positions and sizes of primordia at the fern apex. In Pl. III, Fig. 1, and Text-fig. 5 a photograph and tracing respectively of a large apex of *D. aristata* as seen from above are given. For comparison, Text-fig. 6 is a diagrammatic representation of an apex in which every primordium occupies its theoretically correct position, the angle of divergence being $138\frac{1}{2}^\circ$. The first and most evident point to note is the small size of the more recently formed primordia, P_1 – P_{10} ; it is apparent that in *Dryopteris* there is no question of the leaf primordia constituting a system of touching circles, nor yet of their exercising com-

pressive stress on each other. A second point is that the last primordium to be formed (P_1) is remote from the two previously formed primordia (P_2 and P_3); in fact it lies between and slightly above P_4 and P_6 , being slightly closer to the older primordium (P_6). Again, it would be more accurate to describe I_1 as originating between P_3 and P_5 , rather than between P_2 and P_3 , although the latter description is not incorrect. A third point is that in many of the apices which have been examined the primordia occupy approximately their theoretically normal positions. In some apices, however, while there may be

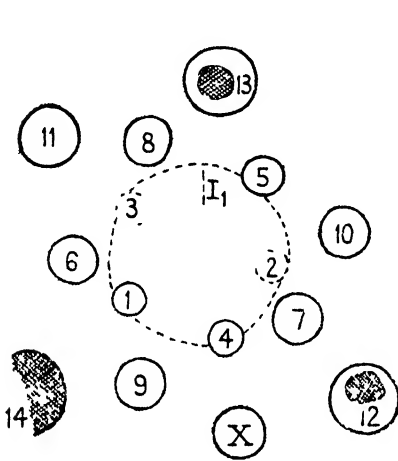


FIG. 5.

TEXT-FIG. 5. Tracing of the photographs illustrated in Pl. III, Fig. 1. I_1 , position of next primordium to be formed; 1, 2, 3, &c, primordia in order of increasing age; primordia 12-14 have been damaged, X, additional primordium (See text)

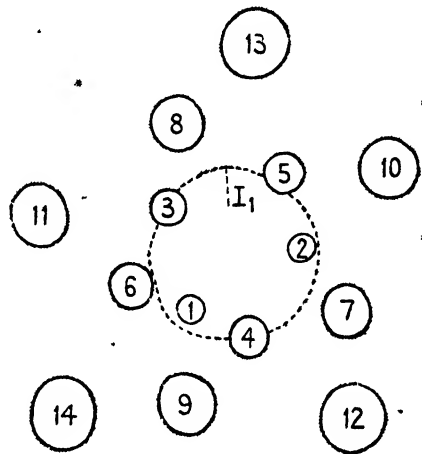


FIG. 6.

TEXT-FIG. 6. An idealized phyllotactic system for comparison with Fig. 5. (· 25.)

a sequence of primordia occupying theoretically correct positions, occasional primordia may arise in aberrant positions, i.e. the angle of divergence may exceed, or be less than, $138\frac{1}{2}^\circ$. In such instances there is not a general displacement of the subsequent phyllotactic sequence: on the contrary, the aberration may remain localized in a particular sector. This can be seen by comparing Text-figs. 5 and 6; P_{11} , P_{13} , P_{12} , and P_9 - P_5 are in approximately their theoretically normal positions and occupy approximately similar positions in the two diagrams; but P_{11} and P_{10} are out of their normal positions, and an additional primordium marked X is present between P_9 and P_{12} . The angle of divergence between P_{11} and PX is approximately $138\frac{1}{2}^\circ$. Text-fig. 5 thus illustrates the difficulty which arises in some specimens of interpreting the age sequence of primordia.

Apices of *D. aristata* were exposed and the position of I_1 observed and marked by a tangential incision in the sub-apical region. Various longitudinal incisions were then made at the base of the apical cone. The specimens on being placed in moist peat formed new primordia at the rate of about one per week.

In one experiment, Text-fig. 7, incisions were made through P_1 , P_2 , P_3 , and P_4 . The new primordia I_1 , I_2 , I_3 , and I_4 which in due course appeared were formed in their normal positions. This result was typical of several experiments of this kind.

When incisions were made in other positions, results of a rather different kind have been obtained, some of these being rather difficult to interpret. Text-fig. 8 shows diagrammatically the positions occupied by leaf primordia at the apex (P_1 , P_2 , &c.) and the positions which would normally be occupied

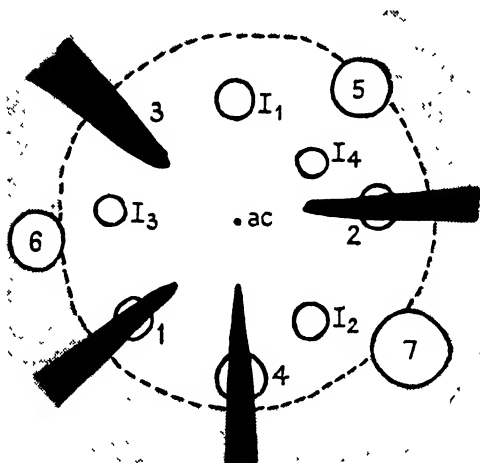


FIG. 7.

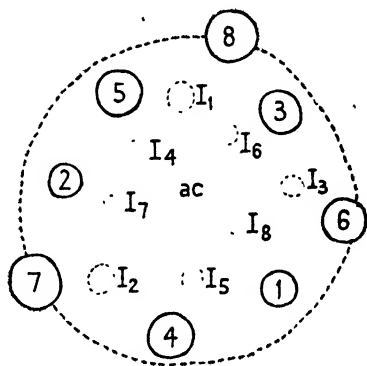


FIG. 8.

TEXT-FIG. 7. Apex which has been incised through primordia 1, 2, 3, and 4. The new primordia, I_1 – I_4 , occupy their theoretically normal phyllotactic positions; *ac*, apical cell. ($\times 20$.)

TEXT-FIG. 8. An idealized phyllotactic system, for comparison with Figs. 9, 10, showing the positions to be occupied by the new primordia I_1 – I_8 .

by the new primordia (I_1 , I_2 , &c.). When such an apex was incised at P_2 , P_3 , P_4 , and close to P_5 , as shown in Text-fig. 9, the new primordia indicated had appeared after 48 days. I_1 (which may have been incipient at the beginning of the experiment) developed in its normal position although its space for development between P_3 and P_5 (or between P_2 and P_3) had been curtailed. I_2 was slightly displaced towards the incision through P_4 . The normal position of I_3 had been obliterated by a superficial scar (*sc*), but a primordium, indicated as I_3 , had been formed in the relatively small space between this scar and the incision through P_3 (Pl. III, Fig. 2). No primordium was formed in the theoretically normal position of I_4 , i.e. between P_2 and I_1 . The next primordium to be formed after I_3 was diametrically opposite (I_4). A bud-like swelling (*b*) was present in proximity to the incision through P_2 . No primordium was present in the normal position of I_5 . The actual position of I_5 is shown: it is situated above and between P_1 and P_6 , instead of P_1 and P_4 . It has, in fact, developed in the widest available space. When a further record was made 3 weeks later, Text-fig. 10, I_6 and I_7

had been formed, both out of their normal positions. I_4 , I_5 , and I_6 make an approximately normal phyllotactic sequence, but the angle between I_6 and I_7 is abnormally small. Moreover, it is evident that while the position of I_7 is related to the immediately adjacent primordia I_2 and I_4 , it is almost completely cut off from the other primordia by the incisions. The relatively limited spaces in which I_1 , I_3 , I_6 , and I_7 have developed raise a question as to the validity of M. and R. Snow's views regarding the spatial requirements for the formation of a primordium. The observations amply confirm their finding

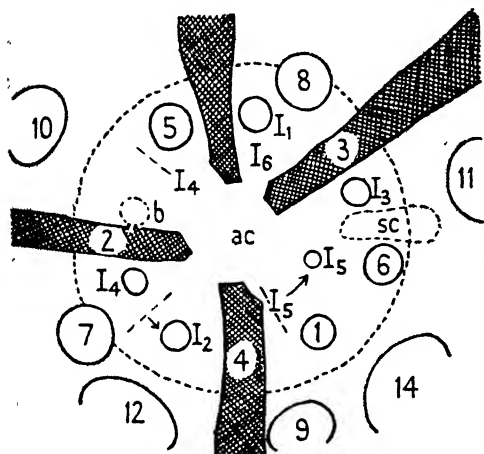


FIG. 9.

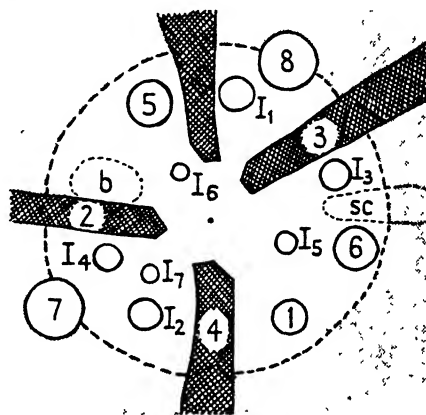


FIG. 10.

TEXT-FIGS 9, 10. Apex incised through primordia 2, 3, and 4, and close to 5. Figs. 9 and 10 show the positions occupied by new primordia after 7 weeks and 10 weeks respectively. *ac*, position of apical cell; *b*, bud; *sc*, superficial scar. (For description see text, and Pl. III, Fig. 2, for photographic illustration.) ($\times 15$.)

that by incising the apex the normal phyllotactic sequence may be seriously disturbed. The position of I_5 is of interest, for although it is considerably removed from its theoretically normal position, it has been formed in the widest space available at the time of its inception. The bud-like development observed during the earlier examination became more conspicuous on further growth, Text-fig. 10; it was evidently not a foliar member. The inferences to be drawn from this experiment are (1) *that the position in which a primordium will arise can be modified*, and (2) *that the position of a new primordium is determined by the local conditions in a sector of the apical meristem and not by conditions in the meristem as a whole.*

In another experiment, incisions were made through P_2 and the position I_1 ; a larger incision was also made between P_1 and P_4 , Text-fig. 11. In this illustration the theoretically normal positions of I_2 , I_3 , &c., are indicated for comparison with the results actually observed, Text-fig. 12. After 51 days, Text-fig. 12, six new primordia had been formed. No primordium was formed at I_1 . I_2 was considerably displaced towards the incision through P_2 , and I_3 towards P_1 . The primordium indicated as I_6' was unmistakably the

youngest present. Two primordia, of approximately equal size and distance from the apical cell, are indicated as I_3 and I_3' . In this specimen the original phyllotactic sequence given by P_3, P_2, P_1, I_1, I_2 comes to an end with I_3 . At this stage of growth the direction of the spiral was reversed, the new sequence, in order of decreasing age and size, being $I_2, I_3', I_4', I_5', I_6'$. This interesting specimen was kept under observation for a further 4 weeks; the four new primordia which appeared were in continuity with the reversed spiral. Here again, by incising the apex, a notable modification of the positions of new primordia has been effected.

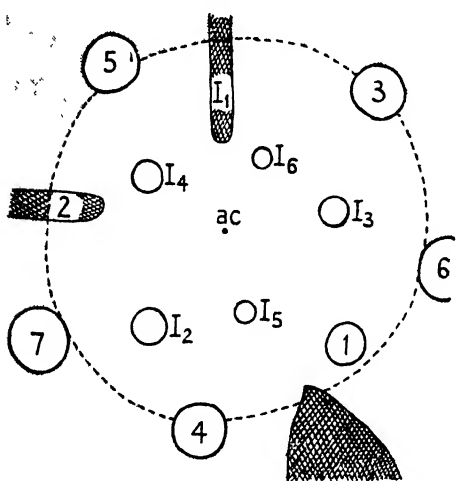


FIG. 11.

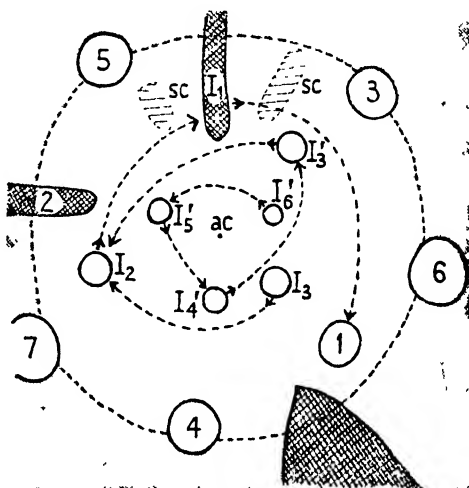


FIG. 12.

TEXT-FIGS. 11, 12. Incised apex showing reversal of phyllotactic sequence; ac, apical cell; sc, superficial scar (For description see text.) ($\times 15$.)

These observations suggest that the position in which a new primordium will arise is not necessarily determined primarily by considerations of space. Thus two primordia, I_3 and I_3' , were formed in the space between incision I_1 and P_1 (or P_3 and P_1), but no primordium was formed in the sector of P_5 , i.e. until I_5' appeared. It may also be noted that I_4' does not occupy a position roughly intermediate between I_2 and I_3 , but is situated appreciably closer to the latter; and I_5' lies much closer to I_2 than to I_3' . Other experimental materials have yielded comparable data. The tendency for a new primordium to be displaced towards an incision, as described by M. and R. Snow, can be seen in the illustrations. Nevertheless, it might perhaps have been anticipated, in terms of the 'widest-available space' hypothesis, that it would be formed in the open space some distance away from the incision.

Further evidence of the effect of incising the apex was sought along the following lines. Apices were incised on either side of P_2 , as illustrated diagrammatically in Text-fig. 13, to see if I_1 and I_2 would be formed in other than their normal positions. If, for example, one or other or both primordia

were formed in positions somewhat removed from the cuts, a system of approximately opposite leaves would be produced. Text-fig. 13 shows the normal positions of I_1 , I_2 , and I_3 ; Text-fig. 14 shows the actual positions occupied by the three new primordia. I_1 was formed in its normal position (it may have been incipient at the beginning of the experiment); I_2 arose some distance away from the incision and from its normal position, and was, in fact, almost diametrically opposite to I_1 , Text-fig. 14. I_3 was in its normal position between P_1 and P_3 , i.e. it was not affected by the incisions on the opposite half of the apical meristem. After a further 26 days the situation was as

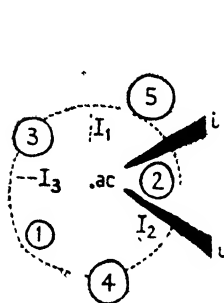


FIG. 13.

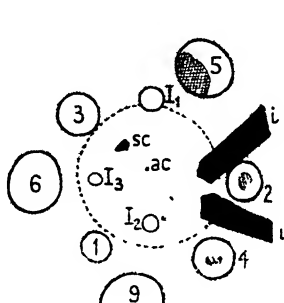


FIG. 14.

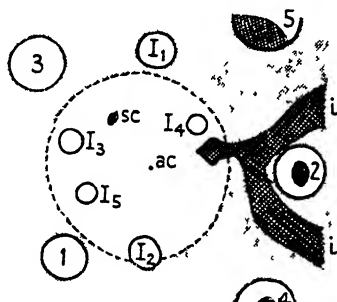


FIG. 15.

TEXT-FIGS 13, 14, 15. Apex incised on either side of P_2 , Fig. 13, the normal presumptive positions of I_1 , I_2 , and I_3 being indicated. Fig. 14 shows the actual positions occupied by I_1 , I_2 , and I_3 , and Fig. 15 the positions occupied by I_1 , I_2 , I_3 , I_4 , I_5 , ac , position of apical cells; sc , small superficial scar, i , incisions. (For description see text) ($\times 12$.)

illustrated in Text-fig. 15. I_4 arose a little out of position, but I_5 , which was formed above and between I_2 and I_3 , was very much out of position. Its theoretically normal position would have been above the axil of I_2 . It will be noted that I_4 and I_5 are almost diametrically opposite. In a second specimen of this series, Text-fig. 16, position I_2 was damaged by the incision, I_3 (labelled I_2') was in its normal position, but thereafter the direction of the phyllotactic spiral was reversed, Text-fig. 17. Several irregular leaf positions are also apparent.

In the experiment illustrated in Text-fig. 18 an incision was made between P_3 and I_1 . No primordium was formed at I_1 ; I_2 was in its normal position, while I_3 was displaced towards the incision and was almost diametrically opposite to I_2 , Text-fig. 19. I_4 , the next primordium to appear, was completely out of position; it may be regarded as being in normal continuity with the *reversed* phyllotactic sequence P_1 , I_2 , I_3 . I_5 , however, stands in no normal relation to I_4 , but is in a normal position relative to I_3 and I_6 . I_4 may also be regarded as being left out of the main phyllotactic sequence. Another possible explanation is that there is a fluctuation between the normal and a reversed phyllotactic sequence. These data are difficult to interpret, the more so as the phyllotaxis of this specimen was anomalous at the outset.

When incisions were made in proximity to I_1 and I_2 as illustrated in Text-fig. 20, I_1 was not formed; I_2 was displaced towards P_2 , i.e. away from the incision; I_3 was in its normal position; I_4 was displaced towards I_1 ,

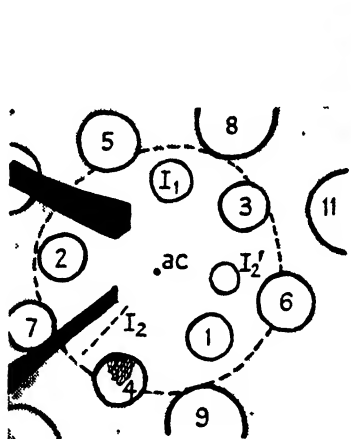


FIG. 16.

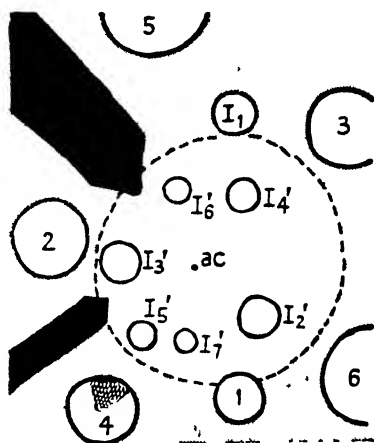


FIG. 17.

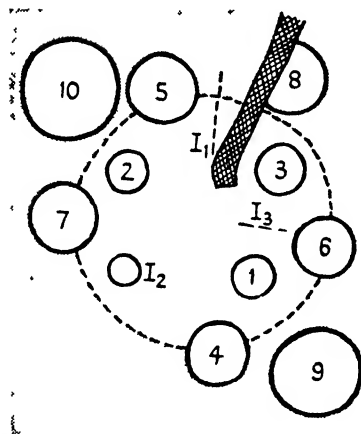


FIG. 18

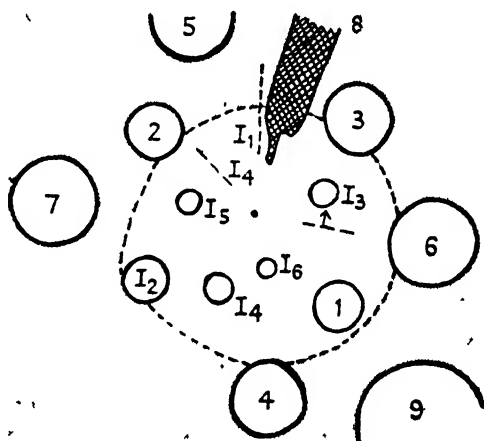


FIG. 19.

TEXT-FIGS. 16, 17. Fig 16: Apex incised on either side of P_2 ; no primordium has been formed at I_2 , and, as Fig 17 shows, the direction of the spiral has been reversed. ($\times 15$.)

TEXT-FIGS. 18, 19. Apex incised close to position I_1 . The presumptive and actual positions of I_1 – I_4 are shown. (For description see text.) ($\times 15$.)

i.e. towards the incision; I_5 was displaced slightly towards the incision; I_6 was in its normal position; I_7 , which should arise almost vertically above P_2 , was considerably displaced towards I_5 (the apex of which had been damaged); I_8 occupied its normal position, Text-fig. 21. In other words, the primordia on the right-hand side of the apex (I_3 , I_6 , I_8) were formed in their theoretically

normal positions: those on the left-hand side—in which the incisions had been made—showed various departures from the normal.

These data show beyond any doubt that the position in which a new primordium will be formed is determined by localized factors and not by factors affecting the apical meristem as a whole. This finding supports the conclusions already reached by M. and R. Snow.

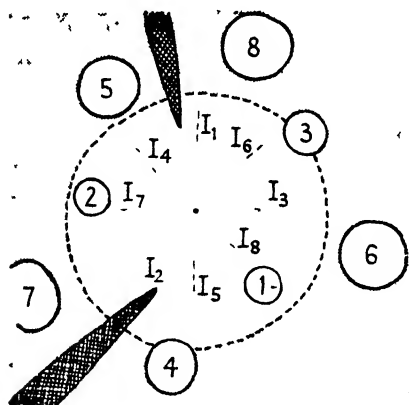


FIG. 20.

TEXT-FIG. 20. Apex incised close to positions I_1 and I_2 . The normal presumptive positions of I_1 - I_8 are indicated.

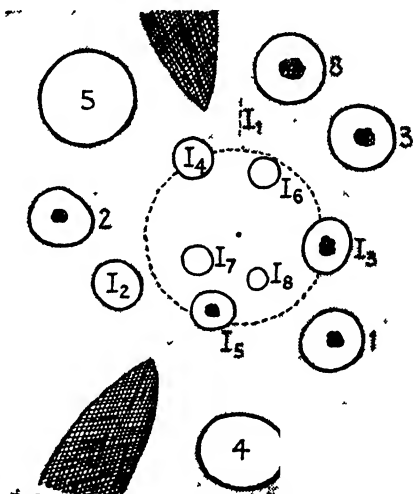


FIG. 21.

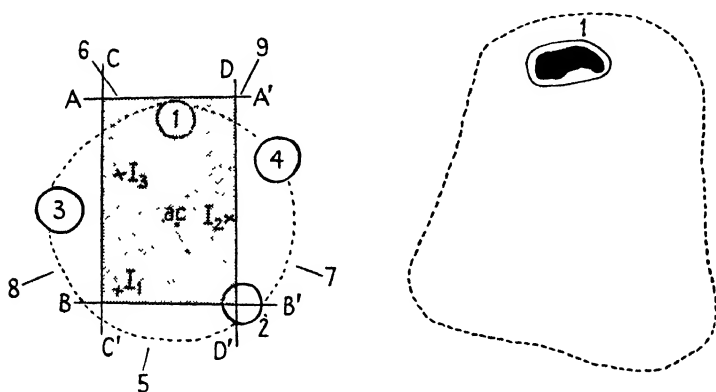
TEXT-FIG. 21. The positions actually occupied by the new primordia are shown. (See text.) ($\times 15$)

These several materials were fixed, embedded in wax, and sectioned transversely. Substantial confirmation was thus available of the various leaf arrangements indicated above.

(c) Leaf formation in laterally isolated apical meristems

The writer has devised a technique by means of which the apical meristem in *Dryopteris aristata* can be isolated by vertical incisions from the adjacent lateral organs and tissues, the incipient vascular tissue being severed by the operation. The isolated meristem, which is seated terminally on a plug of pith parenchyma, is able to continue growth and after some time forms a short vasculated shoot with leaves. In a preliminary account of the observed developments (Wardlaw, 1947) it was noted that the new primordia on the isolated terminal region were in normal phyllotactic continuity with the older primordia in the sub-apical region. Text-figs. 22-9 illustrate the developments observed in an experiment in which the apical meristem was isolated on a rectangular panel together with the last primordium to be formed (primordium 1 in Text-fig. 22). Text-figs. 23-9 illustrate the structural features

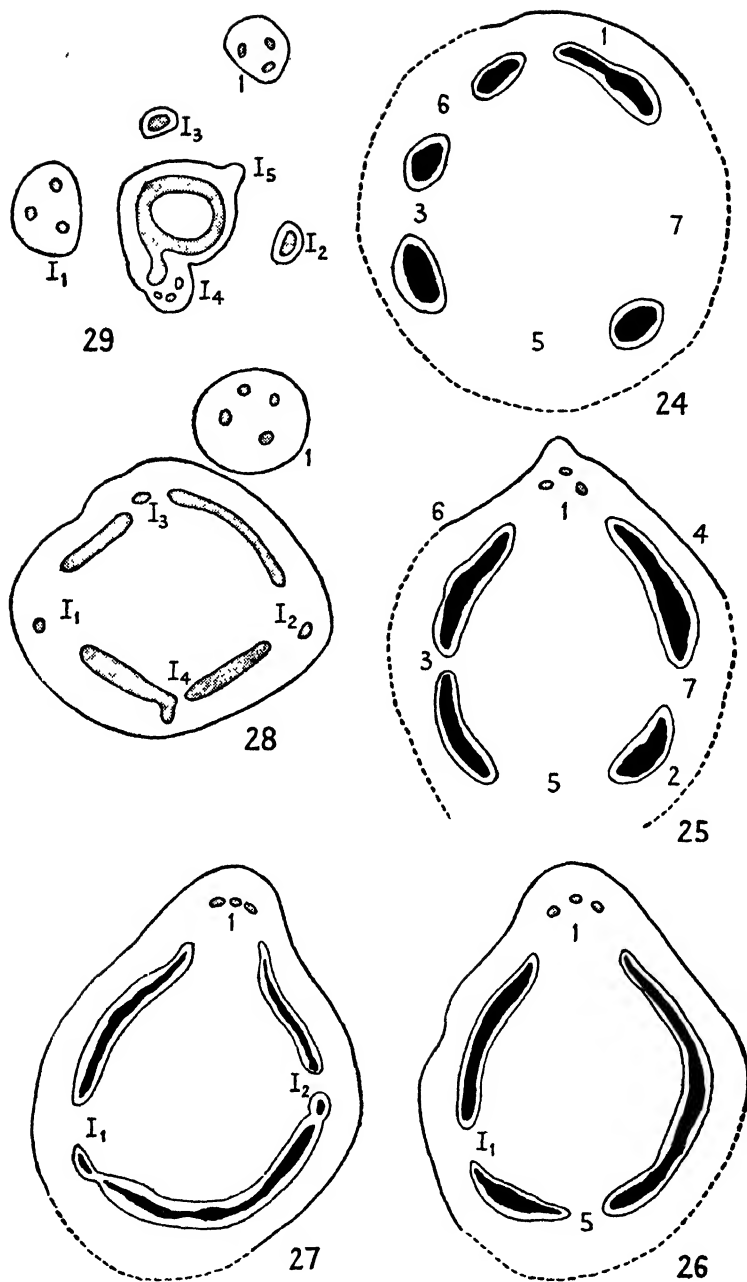
observed in serial transverse sections on proceeding acropetally from below. Near the base of the incisions, the isolated terminal region consists only of pith parenchyma. Higher up a single vascular strand, or meristele, on the side occupied by P_1 , is present, Text-fig. 23. Still higher up other meristeles appear, Text-fig. 24, the gaps between the meristeles being those relating to leaf primordia P_3 – P_7 . As the apex is approached, the leaf-gaps of the older primordia 'close', i.e. there is an apparent conjunction of the meristeles. Here it may be noted that because of injuries to P_2 and P_4 during the incising operation, the gaps of these small primordia remained undeveloped and did



FIGS. 22–3. *See opposite.*

not extend as close to the apex as did those of P_3 and P_5 , the gap of P_5 being more extensive than that of P_3 . The leaf-trace and gap of P_1 —the primordium isolated on the apical panel (Text-fig. 22)—become evident also as the apex is approached, Text-figs. 25, 26. On proceeding upwards, all the gaps of the older primordia (P_2 – P_7) disappear, but new gaps open out, i.e. those of I_1 , I_2 , I_3 , and I_4 , Text-figs. 26–8. These are the newly formed primordia of the isolated terminal region. It can be seen that they occupy normal, or approximately normal, positions with respect to P_1 (see Text-figs. 22 and 28, 29). From these observations it may be inferred that when the apical meristem was isolated so that only the very youngest primordium (P_1) was left, the influence of the older primordia nevertheless remained. The presence of the several wide leaf-gaps extending acropetally supports the writer's earlier findings (Wardlaw, 1947, 1948) that the tangential stresses induced in the axils of the developing primordia extend upwards into the apical meristem, and that such stresses may constitute a factor in phyllotaxis.

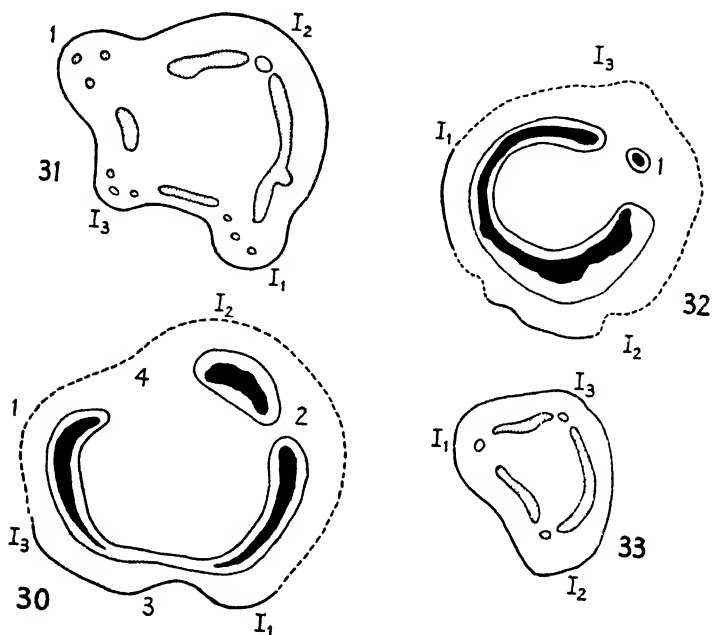
Observations of the kind set out above have been confirmed in many specimens. Two further experiments are briefly illustrated in Text-figs. 30–3. In each instance only the youngest visible primordium (P_1) was left on the apical panel when the meristem was isolated by vertical incisions. In both specimens the vascular system became solenostelic as the gaps of the older primordia—those immediately adjacent to the apical panel—'closed'.



FIGS. 24-9.

TEXT-FIGS. 22-9. Apical meristem and youngest primordium (P_1) isolated on panel by vertical incisions ($AA'-DD'$): this is illustrated diagrammatically in Fig. 22, the presumptive positions of the new primordia being indicated; *ac*, position of apical cell. Figs. 23-9: transverse sections, in acropetal sequence, showing the internal structure (mature vascular tissue with xylem in black; incipient vascular tissue stippled), the 'closing' of the gaps of the older primordia (P_8 - P_7), and the 'opening' of the new gaps relating to I_1 - I_4 . (For description see text.) ($\times 15$.)

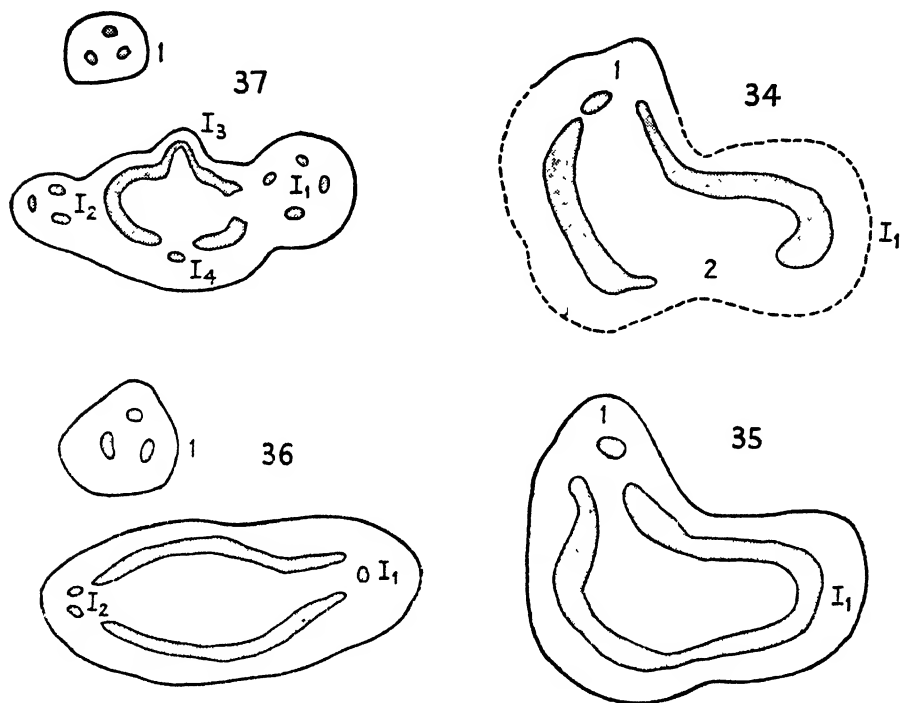
Text-figs. 30 and 32 illustrate the structural arrangements just at or below the insertion of P_1 , the positions of the older primordia and the presumptive or anticipated positions of the new primordia I_1 , I_2 , I_3 being indicated. In sections taken higher up, Text-figs. 31 and 33, the new primordia are seen to occupy the anticipated positions, i.e. they are in normal or approximately normal phyllotactic continuity with the older primordia.



TEXT-FIGS. 30-3. Transverse section, after growth, of two apical meristems, each with its youngest primordium, isolated on a panel. The presumptive and actual positions of the new primordia are shown. Mature vascular tissue with xylem in black; incipient vascular tissue, stippled. (For description see text.) ($\times 15$.)

In another experiment the apical meristem was isolated by three vertical incisions, the primordium P_1 occupying one of the angles of the triangular panel. During the subsequent growth the isolated meristem became somewhat irregular in shape due to a partial collapse on one side, Text-figs. 34, 35. The solenostele which was differentiated in the lower, non-foliar region conformed in its shape with the transverse outline of the new shoot, Text-fig. 35. In this apical meristem the new leaf primordia did not occupy the normal phyllotactic positions: I_1 occupied a position at an angle of approximately 90° to the median longitudinal plane of P_1 , I_2 was diametrically opposite to I_1 ; while I_3 and I_4 were also opposite to each other and at right angles to I_1 and I_2 , Text-figs. 36, 37. From these observations it is inferred that, as a result of the experimental treatment, the distribution of stresses in the apical meristem was very different to that in the normal apex, and this was reflected in the anomalous phyllotaxis. After the appearance of I_2 (Text-fig. 36) the regions

of minimal stress lay between I_1 and I_2 on opposite sides of the apical cone. It was in these positions that the next two primordia were formed. Other examples of diametrically opposite primordia have been described and illustrated in Section 5 (b).



TEXT-FIGS. 34-7. Illustrating anomalous phyllotaxis in an isolated apical meristem, incipient vascular tissue stippled. 1, youngest primordium, isolated on panel with apical meristem. (For description see text.) ($\times 20$.)

(d) *Experiments on leaf formation*

Experiments on leaf formation in *D. aristata* have been described and discussed elsewhere (Wardlaw, 1949). The following conclusions are relevant to the present inquiry. When the apical cell is punctured, so that a minimal amount of necrosis of the apical meristem ensues, the existing leaf primordia undergo further development and new leaf primordia continue to be formed, in normal or approximately normal phyllotactic sequence, until all the available space on the apical meristem is used up. The last of these primordia may thus arise close to the necrosed apical cell. Thus, although the apical cell is essential for the continued growth of the shoot apex, i.e. for maintaining the apical meristem, it does not, apparently, control or determine leaf formation. Leaf formation is independent of the apical cell, being neither activated nor inhibited by it. There is some evidence that the presence of older leaf primordia and leaves may slow down the rate of formation of new primordia, but further investigation is required. The presence of older leaves or

primordia is not directly necessary for the formation of new ones, but indirect effects are not precluded. When small panels of meristematic tissue close to the apical cell are isolated by incisions from the adjacent meristematic tissue, they give rise to leaf primordia and not to bud primordia as might perhaps have been anticipated. Primordia isolated by vertical incisions usually show limited growth and develop into non-laminate, awl-like structures; the dorsiventral symmetry is, however, retained. The chief morphological differences between leaf and bud primordia (see Section 6) can be attributed to the positions

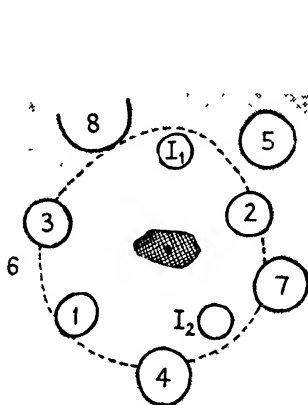


FIG. 38.

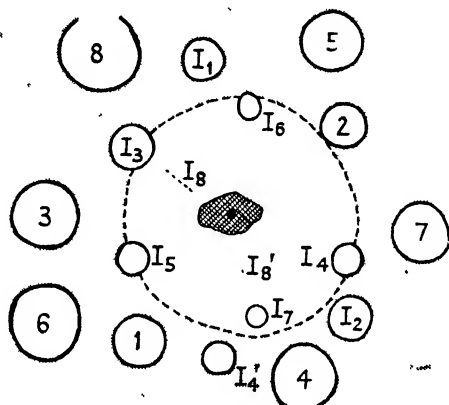


FIG. 39.

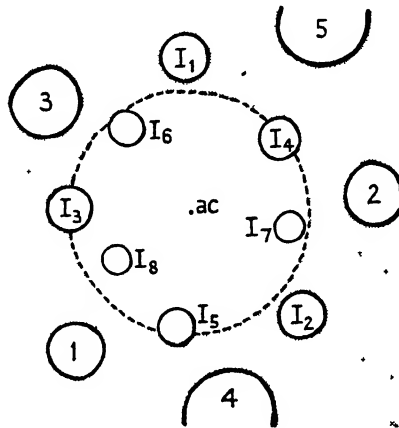
TEXT-FIGS. 38, 39. Formation of leaf primordia, I_1 – I_8 , after the apical cell had been punctured. Compare the positions actually occupied by the new primordia with those in the ideal phyllotactic system illustrated in Text-fig. 40. ($\times 15$)

which they occupy at the time of their formation; all attempts made by the writer to transform very young leaf primordia into buds have so far been unsuccessful.

In Text-figs. 38 and 39 the positions of new primordia (I_1 , I_2 , &c.) after the apical cell had been punctured are indicated. In this specimen the phyllotaxis was already somewhat anomalous at the beginning of the experiment; for example, the angle of divergence between P_4 and P_3 was abnormally small and that between P_2 and P_1 abnormally large. The illustrations show that the necrosed tissue at the apex was somewhat extended along the axis given by P_3 – P_7 . The wide angle between P_2 and P_1 and the small angle between P_1 and P_3 suggests that growth of the apex was more active in the plane of P_3 – P_7 than at right angles to that plane. Text-fig. 38 shows the presence of two new primordia, I_1 and I_2 , after 3 weeks. After a further 5 weeks the record shown in Text-fig. 39 was obtained: in all, nine new primordia were formed. These show several interesting anomalies in the matter of position; for comparison, Text-fig. 40 shows the arrangement in an equivalent ideal phyllotactic system.

I_1 and I_2 occupy normal positions; I_3 is almost diametrically opposite to I_2 ; I_4 is considerably out of position, being abnormally close to I_2 ; an

additional primordium labelled I_4' , which has arisen between P_1 and P_4 , makes a normal angle of divergence with I_3 (with a reversal of the direction of the spiral), but is completely outside the general phyllotactic sequence; I_5 stands in a normal relationship to I_4 , and I_6 to I_5 ; I_7 is not quite diametrically opposite to I_6 ; the anticipated position of I_8 is indicated, but the next primordium to develop, labelled I_8' , is situated between I_5 and I_7 , being rather closer to the latter. The distribution of the new primordia is thus very irregular; whereas five of them are in close proximity to P_4 only one is

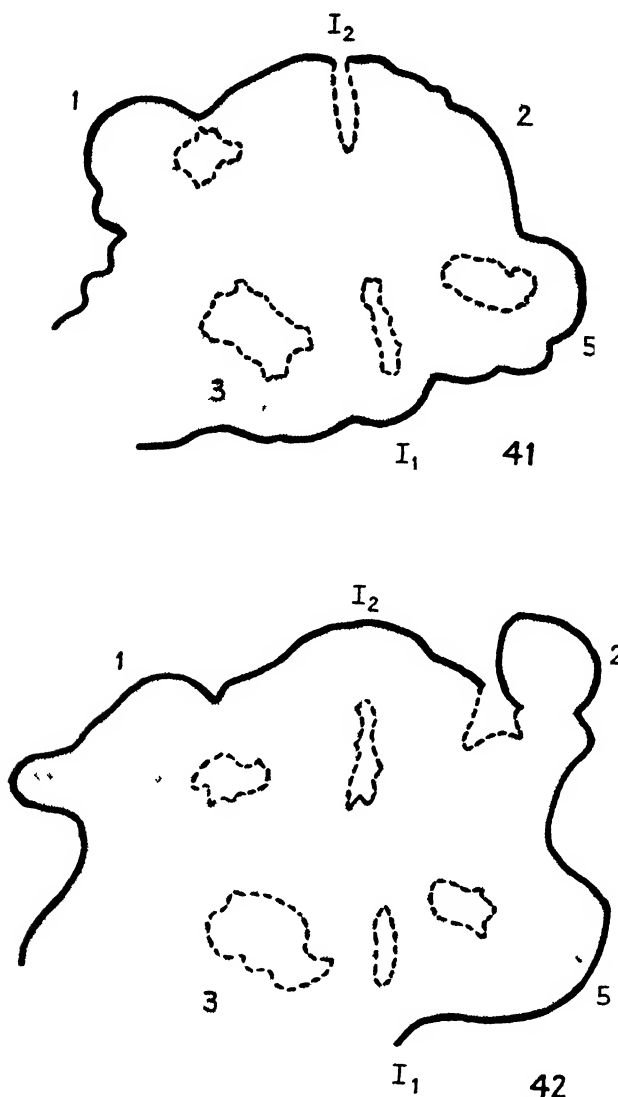


TEXT-FIG. 40. Ideal arrangement of new primordia, I_1 – I_8 , for comparison with Text-figs. 38, 39. ($\times 15$.)

adjacent to P_2 : i.e. in this sector a considerable area of meristem remains unoccupied. In the present state of knowledge it is difficult to explain these data. But they do afford indications that considerable differences may exist in the capacity of different regions of the meristem for growth and morphogenetic activity. The data of Text-figs. 38 and 39 show that some primordia have arisen in the next largest available space, thus giving support to the view of M. and R. Snow, but the very asymmetrical positions occupied by other primordia suggest that these considerations of space will only become meaningful when progress has been made in the investigation of the physiology of meristematic tissue.

(e) Evidence of tensile stress in the leaf axil

Reference has already been made to tensile stress as a factor in phyllotaxis (Wardlaw, 1948). It has been shown that when the axil of a young primordium is incised or punctured the wound becomes tangentially distended: on the other hand, when the positions I_1 and I_2 are punctured, the wounds do not become distended. It has therefore been concluded that new primordia are formed in positions of minimal tensile stress. Incised and punctured apices were fixed, embedded in wax, and sectioned transversely, with the results illustrated in Pl. III, Figs. 3, 4, 5, and Pl. V, Fig. 14. The developments



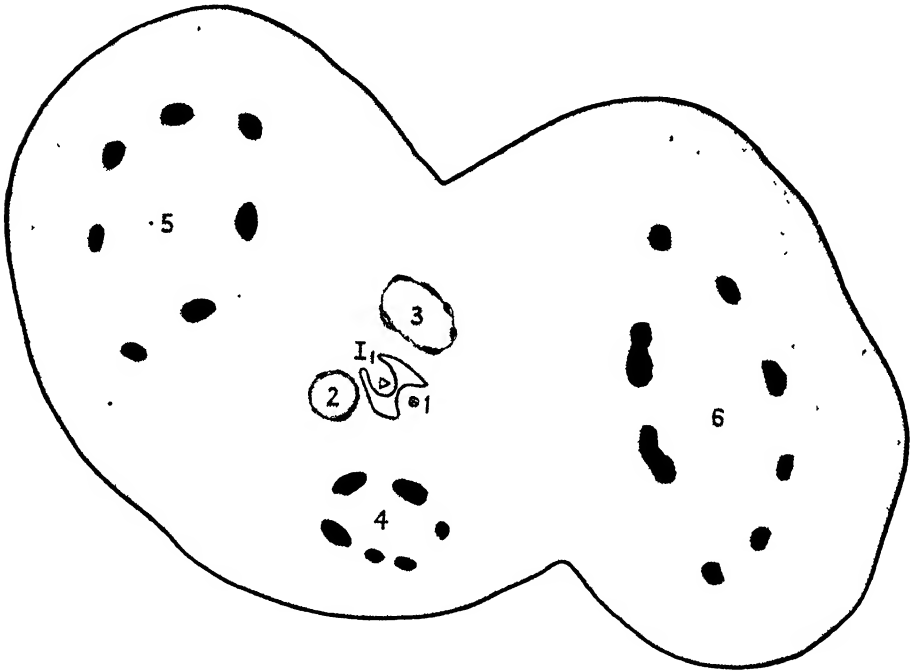
TEXT-FIGS. 41, 42. Tracings of photographs (see Pl. III, Figs. 3 and 4) of transverse sections, at two levels, of an apical meristem which had been punctured in the axils of primordia I_1 , I_2 , 3, and 5, and in positions I_1 and I_2 . ($\times 55$)

observed externally (Wardlaw, 1948) are confirmed by the shapes of the cavities as seen in transverse section. Text-figs. 41 and 42 are tracings of photographs and serve as a key to Pl. III, Figs. 3, 4. The narrow slits in positions I_1 and I_2 are in marked contrast to the wedge-shaped fissures in the leaf axils.

In the specimen illustrated in Pl. III, Figs. 3 and 4, punctures were made in the axils of P_1 , P_2 , P_3 , and P_5 and in the positions I_1 and I_2 . Wide, arrow-shaped cavities have developed in the leaf axils, but the punctures at I_1 and I_2

have remained as narrow slits. The illustrations in Pl. III, Fig. 5, and Pl. V, Fig. 14, afford confirmatory evidence.

The illustration in Pl. IV, Fig. 6, shows an apex, after further growth, in which several incisions were made, the meristem itself and two lateral plugs being isolated by vertical incisions. Pl. IV, Figs. 7–11, illustrate the appearance of typical transverse sections of the apex at different levels: in every instance



TEXT-FIG. 43. Transverse section of the apical region of *Cyathea Manniana* showing primordia 1–6 surrounding the apex. I_1 , position of next primordium to be formed. The triangle indicates the apical cell. The depression surrounding the apical cone is distended tangentially. Mature vascular strands, black; incipient vascular tissue, shaded. ($\times 15$.)

the incisions have widened out. In passing it may be noted that the new primordia I_1 – I_4 occupy normal phyllotactic positions. These observations support the views (a) that as a leaf primordium develops it induces tensile stress in the apical meristem in and above its axil, and (b) that new primordia arise in positions of minimal tensile stress.

In the large shoots of tree-ferns such as *Cyathea Manniana* the apex occupies a slight depression formed by the swollen bases of the adjacent leaf primordia. The shape of this depression is variable. This is due to the very considerable tangential enlargement of the leaf-bases (Wardlaw, 1948a). An examination of such material leaves little doubt that the apical meristem is subjected to tangential tensile stress as each primordium enlarges, the position to be occupied by the next primordium being one in which, from inspection, one would expect tensile stress to be minimal. Text-fig. 43, a transverse section

of the apical region, shows the depression round the apical cone. This depression is markedly distended tangentially in the axils of the recently formed primordia. The position of I_1 is one in which tangential stress may be expected to be minimal.

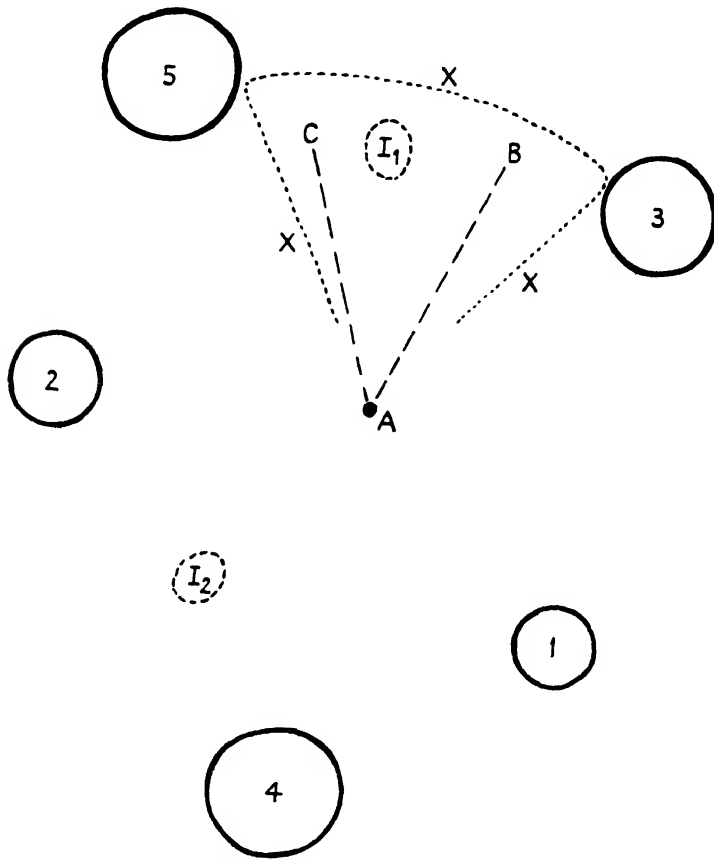
6. ANATOMICAL STUDIES OF LEAF FORMATION

In standard texts and other works on the morphology of leptosporangiate ferns (Hofmeister, 1868; de Bary, 1884; Bower, 1923; Campbell, 1940; Sifton, 1944; Wardlaw, 1945*a*) the leaf is described as originating from a single enlarged cell of the apical meristem. Accordingly, it has been held that in its origin the fern leaf differs notably from the leaves of flowering plants, for in them the primordium is essentially a mound-like outgrowth of the shoot, and a considerable number of cells is involved (see Foster, 1939, and Majumdar, 1945, for reviews of the literature). Notwithstanding these anatomical differences, the evident fact is that the leaves in the two groups have much in common. These considerations led the writer to undertake a re-examination of the earliest stages of leaf formation in ferns. The results have been reported in detail elsewhere (Wardlaw, 1949), but some relevant points may be summarized here. Briefly, it has been ascertained that the very young primordium originates *not* from a single superficial meristematic cell, but from a group of six to eight equivalent meristematic cells. Three or four of these enlarging meristematic cells can be seen in a longitudinal median section of an incipient primordium, Pl. V, Figs. 12 and 13; the underlying cells are also seen to be in a state of active division. At an early stage, however, one of the more centrally placed superficial cells begins to enlarge and soon becomes the conspicuous apical cell of the primordium. The initial stages of leaf formation in flowering plants and ferns are thus, in the main essentials, closely comparable.

7. DISCUSSION

Views on phyllotaxis which are opposed by experimental and other data will be considered first. The writer's observations (Section 5 (*a*)) support the contention of M. and R. Snow (1947) that the position in which a leaf primordium will arise is not determined by the presence of a pre-existing leaf trace. Nor is the view that the superficial layer of the shoot apex grows more rapidly than the tissues within, and so forms folds which become leaves, borne out by the data of the present investigation. In ferns, leaf formation results from the growth of a strictly localized group of superficial prism-shaped cells of the apical meristem together with the underlying cells. Both the experimental and anatomical data are contrary to the view that the primordium is in the nature of a fold of tissue: it is a localized outgrowth of meristematic tissue. At an early stage an apical cell is differentiated and all the subsequent development of the primordium can be referred to the growth and division of this apical cell and its products. Among earlier investigators (Schwendener, 1878; Goebel, 1900) it was held that contact pressures between rapidly

developing primordia constituted a factor which determined the positions of new primordia. Church (1904) has dealt very thoroughly with this and related hypotheses and has shown that while flowering plants afford ample evidence of contact pressures among the older lateral members, very young



TEXT-FIG. 44. Diagram illustrating the positions of primordia 1-5, and the positions of I_1 and I_2 , when the angle of divergence is $138\frac{1}{2}^\circ$. The discontinuous line XXX indicates the space within which I_1 may be formed. AB is equidistant between P_1 and P_2 , AC between P_2 and P_3 . A , position of apical cell.

leaf primordia are widely spaced and quite separate one from another. In this connexion he has particularly mentioned and illustrated the apices of *Nymphaea* and *Dryopteris*. The young primordia in ferns are widely spaced and small relative to the area of the sector in which they arise, Text-fig. 44. The view of Schwendener (1895) and van Iterson (1907) that the insertion of primordia on the shoot apex constitutes a system of touching circles, or, as Goebel (1900, p. 77) puts it, 'that the young organs are laid down in contact with the older', obviously does not apply to leptosporangiate ferns: this point was noted by van Iterson in describing *Matteuccia struthiopteris*.

Consideration may now be given to the hypotheses of Hofmeister and M. and R. Snow and to the interpretation of the data submitted here. Hofmeister's idea, that in different regions of the apical meristem there are differences in the elasticity of the outermost walls, still awaits investigation. But it has been shown that the regions which Hofmeister postulated as having maximal elasticity are those in which tensile stress is minimal. These regions are in marked contrast to the leaf axils where the rapid enlargement of the primordium induces tensile stress. The evidence is that this stress is not confined to the superficial layer or its outer wall: it extends inwards and affects the more deeply seated tissues also (Wardlaw, 1944, 1945). Accordingly, the induction of tensile stress depends on the distribution of growth in different regions of the meristem and in the leaf-bases. A more adequate knowledge of morphogenetic processes will, therefore, depend on a critical investigation of the factors which determine the distribution of growth at the apex.

The strictly localized position and small size of the incipient primordium in *Dryopteris* suggest either the presence of a localized growth stimulus or a localization of cells competent to grow out and form a lateral member, rather than a general protuberance of tissue in a region of low resistance to outward growth.

Hofmeister stated that the new primordium will appear in that position on the apex which lies farthest from the lateral margins of the last-formed primordia. This hypothesis and its interpretation have been considered in detail by M. and R. Snow (1934). Hofmeister's Law applies to the ferns in the general sense that the new primordium will normally occupy a position remote from the last-formed primordia. Thus, in Text-fig. 44 the next primordium to be formed (I_1) is remote from P_1 and P_2 ; but it is neither diagonally opposite to P_1 (the last-formed primordium) nor yet approximately equidistant from P_1 and P_2 (indicated by the line AB); nor does it lie in an approximately intermediate position between P_2 and P_3 (indicated by the line AC). The evident account of its position is that it lies above and between P_3 and P_5 and is rather closer to the older primordium. In a phyllotactic system of this kind some investigators would no doubt regard P_2 and P_3 as the 'contact' primordia. But direct observation of fern apices indicates that the primordia immediately adjacent to I_1 are P_3 and P_5 .

The experimental and observational data for *Dryopteris* presented here fully support the conclusion of M. and R. Snow that the position of a new primordium is affected by *the immediately adjacent primordia*, and not by those which are remote from it. Indeed, in *Dryopteris*, Text-fig. 44, it is difficult to see how I_1 could be affected by P_1 or P_4 . M. and R. Snow have demonstrated that the next primordium to be formed will arise in *the largest gap* above and between the previously formed primordia, its exact position within this space being determined by the shapes and positions of the primordia which border the gap. In Text-fig. 44 the largest gap lies in the sector of P_3 and P_5 . By following the experimental methods of M. and R. Snow it has

been shown that if the apex of *Dryopteris* is longitudinally incised at certain points the positions of new primordia can be modified, i.e. primordia can be induced to appear in various anomalous positions and in some instances the direction of the phyllotactic spiral may even be reversed.

As each primordium develops it sets up a tangential tensile stress in the apical meristem in and above its axil. We do not know the magnitude of these stresses or how extensive are the areas of the apical meristem affected by them. Nor do we know how and to what extent the system of stresses is modified when the apex is incised. But that it is modified can scarcely be doubted. When incisions were made in proximity to the presumptive positions of new primordia, the primordia subsequently formed usually showed some displacement from their normal positions: incisions remote from the presumptive positions, on the other hand, had little or no effect. When longitudinal incisions were made through all the primordia round the apical meristem (i.e. P_1 – P_5), the new primordia, I_1 , I_2 , and I_3 , appeared in their normal positions, i.e. the incisions had an effect comparable with that normally exercised by the growing primordia.

The hypothesis that the induction, by developing primordia, of tensile stress in the apical meristem is a factor in phyllotaxis is supported by a considerable body of evidence. The further hypothesis may be advanced that the system of stresses determines or defines the area of the apical meristem in which a new primordium will arise; for, as we have seen, the widest gap between and above the last-formed primordia is also the region of minimal stress. The implication is that, for leaf formation, a certain minimal area of the apical meristem, in which tensile stress is slight or absent, is essential; and this area must be near the basiscopic margin of the meristem. In the relatively large fern apex, although the young primordium only occupies a small part of this area, its position in it is apparently very precisely determined. As each new primordium is formed in a similar, slightly asymmetrical position between two older primordia, the primordia collectively form a regular phyllotactic system which can be given mathematical expression. In other words, the genetic spiral is a secondary phenomenon—a conclusion which is implicit in the works of those who have given general adherence to Hofmeister's views. Goebel (1922), in studies of leaf development, has called attention to the repetitive nature of the pattern laid down in the formative region, the units of the pattern showing a remarkable constancy of scale at the time of their inception.

At this point it is relevant to consider what is known of the effect of tensile stress on the superficial meristematic cells of the fern apex. The writer (Wardlaw, 1944, 1945, 1947) has already given experimental and other data which admit of the view that when incipient vascular tissue is subjected to tensile stress it is transformed into parenchyma. There is evidence that comparable effects may be produced by tensile stress on the distinctive prism-shaped cells of the apical meristem. These cells do not extend an equal distance down the apical cone on all sides; as shown in Text-fig. 1, they

extend for the least distance in the sector of P_6 and for the greatest distance in the sector of I_1 . The basiscopic margin of the apical meristem is thus denoted by a sinuous line which curves upwards away from the axils of the several primordia (P_1 – P_6) which immediately surround the apex. In and above the primordium axil, where tensile stress is developed, the prism-shaped cells soon lose their meristematic character and become transformed into parenchyma. This finding is supported by other observations. In *Onocleoid* ferns, superficial areas of meristematic tissue—described as bud rudiments or detached meristems which originally formed part of the apical meristem—are separated from it during the growth of the leaf-bases and the shoot. Anatomical studies indicate that these detached meristems occur in those regions of the shoot which have been subjected to minimal tensile stress during the enlargement of the leaf-bases (Wardlaw, 1943, 1943a). The small size of these detached meristematic areas indicates how extensive is the parenchymatous development of the original meristematic cells. These ideas are necessarily tentative; they may even prove to be without validity. Moreover, it is not suggested that tensile stress is always, or the only factor, involved in the formation of parenchyma; other factors, i.e. metabolic factors, are evidently concerned. But in some instances tensile stress may be one of the factors at work.

While it is suggested that the system of stresses at the apex may determine the position in which a new primordium can arise, its actual formation requires further consideration. On this question there are two schools of thought: there are those who regard leaf formation simply as an expression of apical growth; and those who hold that it is due to the action of a specific morphogenetic substance. As we have seen, in Hofmeister's view, provided an area of the apical meristem has outer walls of sufficient elasticity, then a leaf primordium will emerge. Goebel (1900) also regards every part of the meristem as potentially capable of leaf formation. According to M. and R. Snow (1948):

'On the theory of the first available space one need not assume any special causes of leaf formation acting at any special positions on the apex; for all the superficial tissue of the apex is supposed to tend to form leaves as soon as it has undergone the changes involved in moving downwards far enough from the extreme tip. But the determination and formation of a leaf is supposed to need some minimum available space, and in this way the position of each leaf is determined. . . . We agree that in some species with phyllotaxis systems of other than the usual spiral kinds other factors determining leaf positions come in besides the space-occupying process, for instance, in many species with whorled phyllotaxis (Snow, 1942) and in ridge-forming succulents (Weisse, 1904).'

Reference is made to the work of Magnus (1906) on experiments on regeneration in mushrooms: Magnus showed that any point on the surface of the wound tissue was capable of forming a gill rudiment, provided a certain minimal space was available. 'Magnus even suggested that a similar idea might well be applied to phyllotaxis and tested experimentally and he made

some instructive comparisons with leaf formation, especially concerning the determination of a primary area before a rudiment arises from it' (M. and R. Snow, 1948).

It will be seen that in this matter there is a broad similarity between the views of Hofmeister and M. and R. Snow. The same idea underlies the views of Schuepp (1914) and Priestley and Scott (1933). But there are botanists who look for more specific causes of leaf formation. For example, it may be asked why the lateral member should be a leaf and not a bud primordium. The significance, in physiological terms, of the minimal space required for the formation of a primordium, and of the remoteness of the leaf position from the vertex of the meristem, also require further investigation. In the present state of knowledge these questions cannot be answered, but they should be raised and discussed in the light of the available information.

Schoute (1913) has suggested that the centre of a leaf is determined first, the leaf being subsequently organized round this centre; and that a specific substance is produced by incipient leaf primordia which inhibits the formation of others in immediate proximity: hence new primordia arise in the gaps between the older ones. He has also suggested that new leaf centres may be inhibited by substances proceeding from the main apex, these substances being different from those produced by the leaf primordia. Richards (1948) has remarked that geometrically the point of intersection of equal or equivalent 'dispersion circles', relating to two leaf centres in the cycle below, becomes the new leaf centre, but points out that, as this conception fails to account for the transition from one Fibonacci system to another, subsidiary hypotheses become necessary. On theoretical grounds which are considered in some detail, Richards considers that 'the thesis that a new primordium arises round a centre determined as a peak of potential in a field dependent on older primordia appears, on various grounds, to be a more acceptable basis of a general theory than the purely geometrical hypotheses of van Iterson'. In his view the observed arrangement of primordia at the fern apex and their mode of formation afford a strong argument in favour of a field theory of the determination of leaf centres. As to the nature of the determining field he admits that nothing is yet known, 'though presumably it is largely one of diffusion'.

The physiological changes undergone by the prism-shaped cells as they move away from the apical cell during growth are evidently important. In appearance and shape the meristematic cells remain unaltered until they are well down the sides of the apical cone when, as a result of divisions by periclinal walls, they become almost equidimensional. In the ferns the apical cell (and possibly its immediate segments) possesses special physiological properties. In experiments in which the apical cell alone was punctured (Section 5 (d)) leaf primordia continued to be formed until all the available space on the meristem was used up; lateral buds also developed, some on the apical meristem (Wardlaw, 1949). The physiological dominance usually attributed to the apical meristem is thus seen to be exercised by the apical cell. Although the inhibiting action of the apex on lateral buds apparently does not apply to

leaf primordia, it may be that they too are liable to be inhibited by high concentrations of growth-regulating substances proceeding from the apical cell; hence their position, remote from the apical cell.

In the ferns, buds and leaves both originate from quite similar groups of superficial meristematic cells; but whereas buds are more or less completely inhibited by the apical cell, leaf primordia are not. Considerations such as these suggest that the problem of leaf formation is probably more complex than M. and R. Snow have suggested.

Evidence of the existence of leaf-forming substances is still very scanty. The hypothesis that such substances do exist raises the question of their source. Their strictly localized action also requires explanation. M. and R. Snow (1937) have shown that if heterauxin in lanoline is applied to a part of the shoot apex in certain flowering plants, the growth of the existing leaf primordium and its bud, or of those which subsequently arise, is promoted. Ball (1944) found that when indole-acetic and indole-propionic acids were applied to the shoot apex of *Tropaeolum majus* the densely cytoplasmic cells of the embryonic region gave no response. But the shoot apex did respond by manifesting various abnormal developments; among others, foliar primordia appeared out of their normal positions, the changes observed being entirely erratic. After a short period of abnormal growth the apex became normal again. Ball concludes that the shoot apex is an independent, self-determining region of the plant. In a study of rye embryos, de Ropp (1945) has shown that when excised stem tips were placed in a culture medium there was evidence of growth in the first leaf only. He observed, however, that if any isolated stem tip developed a root the whole growing-point was stimulated to meristematic activity and leaves developed. In the researches of Went (1938) and of Bonner, Haagen-Smit, and Went (1939, 1939a) certain substances, including auxin, are seen to be important in leaf growth; but there is little information on substances necessarily involved in the initial stage of leaf formation. Went (1938) has developed the concept of 'calines', by which he means specific biochemical factors for organ formation other than auxins. Auxin, though necessary for growth, is not the only growth factor involved in the formation and development of the several organs. Other hormone-like substances, rhizocaline, caulocaline, and phyllocaline, are held to be necessary for the formation of roots, shoots, and leaves respectively, these substances moving through the living cells. Phyllocaline, according to Went, is stored in the cotyledons of flowering plants; it is formed in leaves in the light but is not present in the stem to any extent. As soon as the cotyledons are removed leaf growth ceases. Went admits that the existence of calines has not so far been directly proven, 'but enough evidence has been collected to make their existence highly probable. In addition, the assumption of the presence and action of such calines offers the simplest explanation of many other effects related to growth and development of plants.' From experimental observations of a rather different kind Gregory (1928) also concluded that a special factor necessary for leaf growth is formed in the older leaves under the

influence of light. This factor is not directly related to carbohydrate photosynthesis. Certain experiments by Le Fanu (1936) and Snow (1936) also indicate the action of a factor additional to auxin in growth actions. In morphogenetic studies, using tissue cultures, both White (1939) and Skoog (1944) have rejected Went's 'caline' hypothesis as unnecessary. In cultures which had been maintained in the undifferentiated condition for long periods and during many transfers they were able, by appropriate experimental treatment, to induce the development of leafy shoots and roots. What then, they ask, was the source of the caulocaline and phyllocaline?

As applied to the ferns, Went's hypothesis also presents difficulties, but the experimental evidence so far obtained does not lead to its total rejection. Ferns do not, of course, possess storage cotyledons in which the initial supply of phyllocaline might be stored. Moreover, the removal of adult leaves and of all young leaf primordia over long periods does not prevent the formation of new primordia (Wardlaw, 1944). And again, apical meristems isolated from young and older leaf primordia by vertical incisions (Wardlaw, 1947, 1949) continue to form leaf primordia at the normal rate. It could, of course, be argued that phyllocaline is stored in the central pith on which the isolated meristem is seated; this, however, would represent a departure from Went's view that phyllocaline is formed and stored in the leaves and supplied to the stem. Went's hypothesis also does not explain how phyllocaline acts in quite specific positions in the apical meristem.

Albaum (1938) states that the first leaf of the fern sporophyte—the nearest equivalent to a cotyledon in flowering plants and apparently the primary source of hormones in the fern plant—produces a hormone which inhibits the outgrowth of adventitious processes on the parent prothallus and of other sporophytic leaves. If the primary leaf is removed and the cut surface of its petiole is smeared with indole-3-acetic acid in lanoline, a similar inhibition of the growth of the younger leaves is produced. It is not made clear, however, to what extent the formation of new leaves is affected.

Certain observations made by Thimann (1938) indicate that roots, buds, and shoots react to auxins in essentially the same way. They simply differ in the quantities required to produce the effects, roots requiring least auxin, shoots most. Each type of organ is stimulated by a relatively low concentration of auxin and inhibited by a high concentration. In the ferns there is evidence that auxin is produced by the apical cell and moves downwards. From the fact that leaf primordia are formed at the apical meristem, whereas buds are usually formed lower down, it could perhaps be argued that leaf formation is stimulated by auxin at concentrations that inhibit bud formation. But in close proximity to the apical cell, where the concentration of auxin is highest, leaf formation will normally be inhibited. Such a conception would explain why the leaf occupies a position on the meristem as remote from the apical cell as possible, and why, when the apical cell is punctured, primordia may be formed close to the vertex.

It is evident that we are not yet in a position to give any adequate account

of the factors determining leaf formation; and whether or not specific morphogenetic substances are involved must remain an open question. Elsewhere the writer (Wardlaw, 1949) has considered in some detail the question of leaf and bud formation.

In the literature on leptosporangiate ferns the leaf primordium has been described as originating from a single, prism-shaped cell of the apical meristem (Hofmeister, 1868; de Bary, 1884; Bower, 1923; Sifton, 1944; Wardlaw, 1945*a*), this cell rapidly enlarging to the characteristic 'two-sided' apical cell. It was inevitable, therefore, that the leaves of ferns and of flowering plants—in which the leaf primordium consists of an outgrowing multicellular mound of tissue—should be regarded as organs in which neither homology of origin nor homoplastic development could be assumed. Yet, notwithstanding the apparent difference in the inception of leaves in the two groups, it was evident that the two organs had much in common, e.g. their formation at the apex, phyllotaxis, dorsiventral symmetry, and general organographic development. The new anatomical data summarized here (Section 6) show that, in the earliest stage, the primordia of ferns and flowering plants are in essentials alike, i.e. both consist of multicellular protuberances or outgrowths of the apical meristem.

The general conclusions that have emerged from the investigations described and discussed here are these: (i) Leaf formation, an expression of growth at the shoot apex, is a complex process which will only be more fully understood as our knowledge of the factors controlling growth at the apex are more adequately investigated. (ii) Whether (*a*) the meristematic cells which take part in the formation of a leaf primordium do so because they are in some particular physiological state—as yet unspecified—this state being dependent on their position on the meristem, i.e. remote from the apex, or (*b*) a specific morphogenetic substance is involved, cannot be decided on the evidence so far available. Should it be shown that a morphogenetic substance is involved, its strictly localized action will still have to be explained. (iii) The next primordium to be formed normally arises on the widest available gap above and between the last-formed primordia, its position within that gap being determined by the primordia which border it; while it may be held that a certain minimal space is necessary for a leaf primordium to arise, it should be noted that in *Dryopteris* the primordium only occupies a small fraction of the space available. (iv) As each new primordium arises with a high degree of constancy in a characteristic position within the 'first available space', the formation of successive primordia is seen to constitute a phyllotactic system of a high order of regularity. (v) A single primordium can be out of its theoretically normal position without disturbing the subsequent phyllotactic sequence, i.e. the aberration is strictly local. (vi) There is evidence, both experimental and observational, that the position in which the next primordium will arise—i.e. in the 'first available space' or 'widest gap'—is one in which tensile stress is minimal. (vii) The view is advanced that, in *Dryopteris*, the system of stresses induced in the apical meristem by the existing primordia

may be the factor which determines, or defines, the space in which the next primordium can arise, but other factors are responsible for the actual process of leaf formation.

8. SUMMARY

1. Experimental and anatomical investigations of phyllotaxis and leaf formation in ferns are described and discussed.

2. Several hypotheses advanced by other workers are rejected in the light of the data now available: (a) that the position in which a primordium will arise is determined by the presence of a pre-existing leaf-trace; (b) that the superficial layer of the meristem grows more rapidly than the underlying layer and so forms folds which become leaves; (c) that contact pressures between rapidly developing primordia constitute a factor which determines the positions of new primordia; (d) that the insertion of primordia on the conical shoot apex constitutes a system of touching circles.

3. Hofmeister's view, that the new primordium will appear in that position on the apex which lies farthest from the margins of the last-formed primordia, is acceptable in the general sense that the new primordium will normally occupy a position remote from the next older ones. But the hypothesis of M. and R. Snow that the new primordium will occupy the first available space—i.e. a space on the apical meristem of a certain necessary width and a certain distance below the extreme tip—and that the position of the new primordium within this space is determined by the primordia bordering it, undoubtedly affords a more accurate conception of the phenomenon of phyllotaxis. It is borne out by experimental and other observations in the ferns as well as in the flowering plants. The position of the new primordium is determined by the primordia *immediately adjacent* to its presumptive position and not by those remote from it. But since, in the normal apex, the new primordium arises with a high degree of constancy above and between older primordia, the sequence of primordia constitutes a regular phyllotactic system.

4. In *Dryopteris*, as in flowering plants, it has been shown that by incising the apex in certain positions the positions of new primordia can be modified and the direction of the phyllotactic spiral may even be reversed.

5. It has been shown that as each primordium develops it induces a tangential tensile stress in and above its axil; but the position in which the next primordium will arise is one of minimal tensile stress.

6. The factors which determine leaf formation and phyllotaxis are discussed and the conclusion is reached that both physical and physiological factors must be considered. It is held that the system of stresses induced in the apical meristem by the existing primordia determines, or defines, the space in which the next primordium will be formed, but other factors are responsible for the actual process of leaf formation. Whether (a) leaf formation is simply an expression of the 'tendency' of the apical meristem to grow, (b) the meristematic cells which form a leaf primordium do so because they are in

some particular physiological state, or (c) some specific 'leaf-forming substance' is at work, cannot be decided on the evidence so far available.

7. Anatomical evidence is summarized which shows that in the earliest stage of leaf formation in *Dryopteris* a group of superficial cells is involved and not a single meristematic cell as had previously been thought. The leaf primordia of ferns and flowering plants are thus seen to agree closely in origin as in many other respects.

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EXPLANATION OF PLATES

Illustrating Professor C. W. Wardlaw's paper on Leaf Formation and Phyllotaxis in *Dryopteris aristata* Druce

(All figures are from untouched photographs)

PLATE III

Fig. 1. Downward view of a large shoot apex showing the sequence of leaf primordia; see Text-fig. 5. ($\times 40$.)

Fig. 2. An incised apex showing new leaf primordia which have developed between, and in proximity to, the incisions; see Text-figs. 9 and 10. ($\times 40$.)

Figs. 3, 4. Approximately transverse sections, at two levels, of an apex which had been punctured in the axils of leaf primordia P_1 , P_2 , P_3 , P_6 , and in the positions I_1 and I_2 . The punctures in the axils have been distended tangentially: those in the positions to be occupied by the next two primordia, I_1 and I_2 (centre, below, and above), have remained narrow and slit-like; see Text-figs. 41 and 42 for key. ($\times 70$.)

Fig. 5. Transverse section of an apex which had been punctured as in Fig. 3: top right, P_3 ; top left, P_6 ; top centre I_1 . The punctures in the leaf axils have opened out into wide, wedge-shaped fissures: that at I_1 has remained narrow and slit-like. ($\times 70$.)

PLATE IV

Fig. 6. Drawing of an apex, as seen from above, which had been incised in the axils of P_1 – P_8 (P_6 excepted). The apical meristem was also isolated laterally from the adjacent organs and tissues by vertical incisions; two plugs supporting leaf primordia were also isolated. The new primordia I_1 – I_4 have been formed in approximately normal phyllotactic sequence. ($\times 25$.)

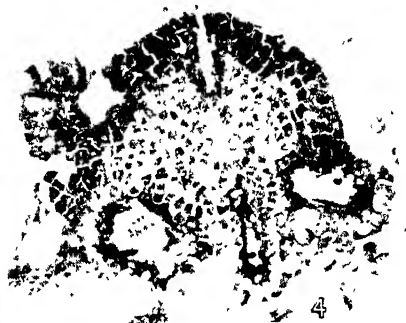
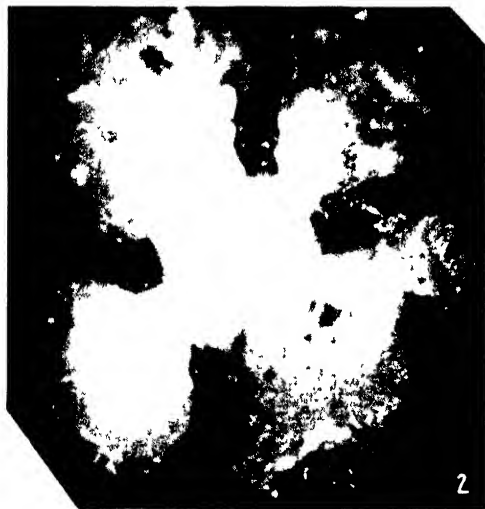
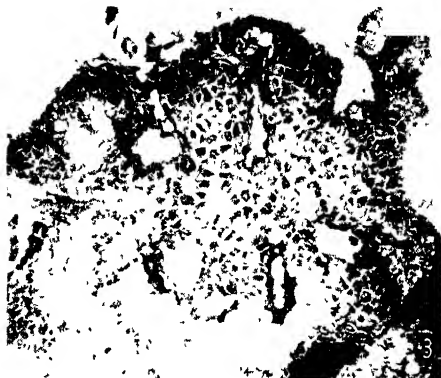
Figs. 7–11. Serial transverse sections, in basipetal sequence, of the apex shown in Fig. 6. Without exception the incisions through the leaf axils have widened out into wedge-shaped fissures. Fig. 11 illustrates the approximately solenostelic condition in the sub-apical region. ($\times 25$.)

PLATE V

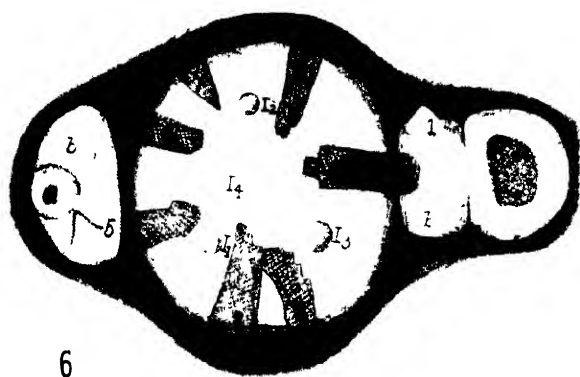
Fig. 12. Longitudinal median section of a very young leaf primordium. Several of the superficial cells of the apical meristem have enlarged to form a slight protuberance; the underlying cells are also in a state of active division. The apical cell of the shoot is to the left. ($\times 350$.)

Fig. 13. As in Fig. 12: the slight protuberance, which denotes the earliest stage of leaf formation, consists of several enlarging superficial cells of the apical meristem. Apical cell of shoot to the right. ($\times 350$.)

Fig. 14. Transverse section of a shoot apex which had been incised in the axils of leaf primordia P_1 and P_3 . Wedge-shaped fissures have developed; P_4 on left. ($\times 70$.)



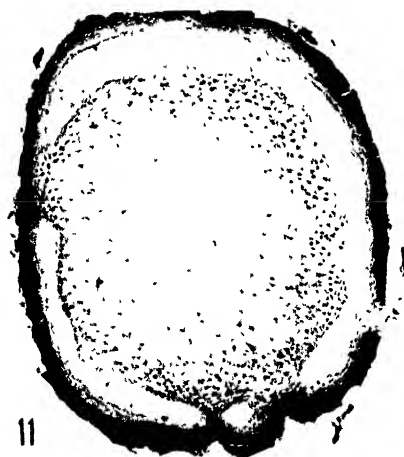
WARDLAW—FERN PHYLLOTAXIS



6



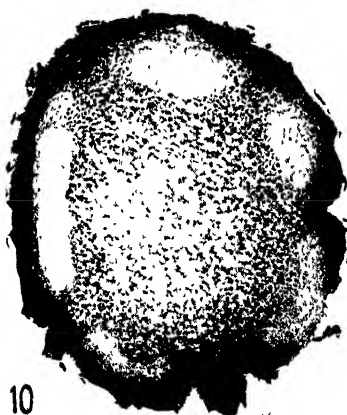
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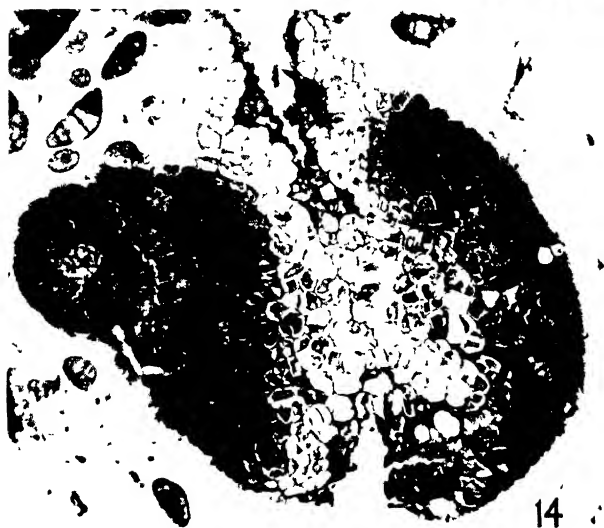
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9



The Carbohydrate Nutrition of Tomato Roots

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With six Figures in the Text

INTRODUCTION

WHITE (1936, 1940a) found sucrose markedly superior to dextrose as a source of carbohydrate for excised tomato roots. These results are, however, at variance with those of Robbins and Bartley (1937) and Robbins and Schmidt (1938) who, using subcultures supplied by White, reported that dextrose was superior to sucrose, giving more rapid and prolonged growth and whiter roots. The photographs by Robbins and Schmidt (1938, Fig. 16) of excised roots growing on different carbohydrates show differences not only in growth rate but also in general morphology. Subcultures of a clone of excised tomato roots (Best-of-All) established in our laboratory have shown even sharper contrasts between cultures grown on sucrose and those grown on dextrose, and between those grown on yeast extract and those supplied with a synthetic organic supplement. These observations formed the starting-point of the present investigation.

EXPERIMENTAL PROCEDURES

(a) *Excised root-culture technique*

Tomato seeds (Best-of-All) were surface sterilized by treatment for 5 minutes with 1 per cent. solution of bromine in water, washed five times with sterile distilled water, and the seeds transferred singly under aseptic conditions to sterile Petri dishes containing filter-paper moistened with sterile distilled water. The Petri dishes were placed in a germinator and maintained at 25° C. until the radicles were 20–30 mm. long. The initial cultures were then set up, each flask being inoculated aseptically with a single radicle tip 5–7 mm. long. After incubating the cultures for 12–15 days and rejecting any contaminated flasks subcultures were made, each being inoculated with a tip 7–12 mm. long cut from a vigorous lateral root. The clone used throughout this work was derived from a single radicle tip and was maintained by regular subculture on the sucrose-synthetic medium. The growth increments were determined by measuring the increase in length of the main axis after 10 days in culture and are expressed as the mean increase in length in mm. per

culture per day. This method is simply a measure of the rate of elongation of the main axis and gives no indication of the total growth because of the contrast in root diameter and in the extent of branching between roots growing in the different culture solutions employed. Although growth increments were determined after 10 days' growth, a number of cultures in each experiment were retained until growth had apparently ceased and the extent of growth compared with that of roots grown in White's medium (1943) containing sucrose 2 per cent. In determining root diameters each root was mounted on a microscope slide and five diameter measurements taken at intervals along the main axis using a calibrated eye-piece micrometer. Each quantitative experiment designed to compare growth increments for the different culture solutions involved the use of a number of stock cultures. In order that any variability between different stock cultures might be evenly distributed, the subcultures in any one culture solution were derived in equal numbers from each stock culture employed. Not less than thirty comparable cultures per culture solution were used as the basis for calculation of growth increments (except in the cases indicated) and the values submitted to statistical analysis as indicated by White (1943). The cultures studied in *Series I* were incubated at $24^{\circ}\text{C} \pm 1^{\circ}$ and sample 1 of Difco yeast extract used in preparing the yeast media. The cultures studied in *Series II* were incubated at $27^{\circ} \pm 1^{\circ}$ and sample 2 of Difco yeast extract used (see Table I).

(b) *Culture vessels and preparation of culture solutions*

100-ml. wide-necked Hysil conical flasks were used as culture vessels. The flasks were washed with soap and water, cleaned with chromic acid, rinsed six times with tap-water, twice with distilled water, and once with double-distilled water. The double-distilled water was prepared from pyrex-glass stills. Each flask was closed with a non-absorbent cotton plug wrapped in muslin and the plug protected from dust with an inverted 50-ml. squat-type Hysil beaker. The flasks were sterilized in the autoclave at 15 lb. for 15 minutes and charged with 50 ml. culture solution. The plugs were then capped with cellophane and the flasks resterilized. All flasks were used within one month of preparation.

The culture solutions were prepared with double-distilled water and were transferred to the culture flasks and sterilized within 24 hours of preparation. The basic culture solution was prepared according to White's formula (1943), was labelled *Sucrose 2 %—synthetic* (*S 2 % S*), and contained in mg. per litre: sucrose (B.D.H.) 20,000; glycine 3; vitamin B₁ 0.1; nicotinic acid 0.5; vitamin B₆ 0.1; Ca(NO₃)₂ 200; MgSO₄ 360; KNO₃ 80; KCl 65; Na₂SO₄ 200; NaH₂PO₄ 16.5; KI 0.75; Fe₂(SO₄)₃ 2.5; MnSO₄ 4.5; ZnSO₄ 1.5; H₃BO₃ 1.5. The inorganic salts and the glycine were 'Analar' grade. *Dextrose 2 %—synthetic* (*D 2 % S*) solution was prepared in the same way except that dextrose (B.D.H. Analar) 20 g. per litre was substituted for the sucrose. Other synthetic solutions differing from the above either in the concentration or nature of the carbohydrate were prepared and labelled in an analogous manner. A corre-

sponding series of yeast solutions was prepared by substituting for the glycine and pure vitamins a Difco yeast extract at concentrations ranging from 2.5 mg. to 100 mg. per litre (e.g. *S* 2% *Y* 2.5, *D* 2% *Y* 100, &c.). In a few cases small amounts of Difco yeast extract were added to synthetic solutions, e.g. *S* 2% *S* *Y* 2.5 = sucrose 2%—synthetic solution to which has been added 2.5 mg. Difco yeast extract per litre. The pH of the synthetic culture solutions after sterilization ranged from 4.1 to 4.3. The presence of 100 mg. Difco yeast extract per litre raised the pH to within the range 4.7–5.1.

(c) *Seedling culture technique*

Seeds of tomato (Best-of-All), surface sterilized as previously described, were germinated in the dark in a thin layer of sterile pure silica sand supported at the surface of a sterile culture solution. The radicles grew through the sand and entered the culture solution. Sterile seedlings were harvested after 21 days and the portion of the root system which had grown into the culture solution was examined. The media used were (1) double-distilled water; (2) *Sucrose* 2%—*inorganic* (*S* 2% *I*) containing the inorganic salts of White's medium; (3) *Dextrose* 2%—*inorganic* (*D* 2% *I*).

(d) *Anatomical technique*

All material was fixed in acetic alcohol, sectioned at a thickness of 8μ , and double-stained with safranin and aniline blue. In view of the large amount of material which had to be examined the investigation was almost entirely confined to the examination of transverse sections. Measurements and cell-counts required the use of a large number of sections because of the marked variation encountered within any single culture.

It was impracticable to prepare each section from a separate individual root, but care was taken not to use sections cut very close together. The use of the proximal parts of roots, which are commonly of smaller diameter than the rest, was avoided. In all cases the piece of root which had served as the original inoculum was excluded in selecting material for sectioning. In each set of numerical observations only one figure was taken from each section, except in counting the number of layers of cortical cells and in estimating the thickness of the cortex; in this work two or three readings were taken along different radii of each section.

The estimation of the significances of the differences between means has been carried out by means of the *t* test. The number of observations in a set was very variable, depending on the amount of material available, but it was generally possible to arrange that *t* should have not less than 20 degrees of freedom.

(e) *Sugar estimations*

Reducing sugars were determined by the method of Lane and Fynon (1923). Sucrose was determined by the increase in reducing sugars resulting from heating for 2 hours in a boiling water-bath after addition of dilute sulphuric acid to give a concentration of 1 per cent. w/v H_2SO_4 . Laevulose

was determined by the method of Becker and Englis (1941). Dextrose was calculated from the excess of reducing sugars over laevulose.

(f) *Preparation of alcohol-precipitated sucrose, ether-extracted sucrose, and of a concentrate of the alcohol-soluble matter of sucrose*

These products were prepared in order to see if the superiority of sucrose over dextrose arose from the presence in the sucrose sample of beneficial impurities soluble in alcohol or in ether.

Sucrose, 200 g., was dissolved by warming in 100-ml. double-distilled water. To this solution when cold was added, with constant stirring, 1,200 ml. 95 per cent. ethyl alcohol and the mixture set aside in a refrigerator overnight. The precipitated sugar was collected on a sintered glass filter, dried carefully at 60° C., and named *alcohol-precipitated sucrose I*.

The filtrate was freed from alcohol and concentrated to 100 ml. *in vacuo* at a water-bath temperature of 40° C. 600 ml. of 95 per cent. ethyl alcohol was then added, the solution set aside in a refrigerator overnight, and the precipitated sugar collected and dried as described above and named *alcohol-precipitated sucrose II*.

The filtrate was freed from alcohol and concentrated to 30 ml. and then treated with 200 ml. 95 per cent. ethyl alcohol. The precipitate was removed and the filtrate again concentrated (to 20 ml.) and treated with 200 ml. 95 per cent. ethyl alcohol, the second precipitate being removed. The filtrate was then concentrated until it began to crystallize and then taken up in 20 ml. of double-distilled water. This process of concentration followed by dilution was repeated in all three times in order to remove the last traces of alcohol. The residue was finally adjusted to 40 ml. with double-distilled water, filtered, and named concentrate of *alcohol-soluble matter of sucrose* (1 ml. \approx 5 g. original sucrose, contained 43 mg. sucrose).

The *ether-extracted sucrose* was prepared by dissolving 200 g. sucrose in 100 ml. double-distilled water, adding 3 ml. of N acetic acid, and extracting the syrup five times with 25-ml. portions of 'Analar' ethyl ether. The syrup was then freed from ether, treated with 1,200 ml. 95 per cent. ethyl alcohol, stored overnight in a refrigerator, and the precipitated sugar collected and dried as described above.

EXPERIMENTAL RESULTS

Excised Root Cultures in Different Media

1. *Habit, rate, and extent of growth*

(a) *In synthetic culture solutions*

Sucrose 2%—synthetic (*S* 2% *S*): Growth rapid, first-order laterals well developed but second-order laterals rarely formed during first 21 days of growth, roots white during period of active growth, turning pale yellow as cultures aged, growth prolonged so that whole of culture solution became permeated by roots.

- Sucrose 4%—synthetic (*S* 4% *S*): Growth less rapid than in *S* 2% *S*, but habit of growth similar and continuing for a prolonged period.
- Dextrose 2%—synthetic (*D* 2% *S*): Growth extremely slow, laterals hardly ever produced, growth ceasing within a month, roots extremely thin and translucent, tips often curved leading to occasional formation of loops as growth proceeded.
- Dextrose 1%—sucrose 1%—synthetic (*D* 1% *S* 1% *S*): Growth, habit, and appearance very similar to that recorded for *S* 2% *S*, but growth slightly slower and finally less extensive.
- Laevulose 2%—synthetic (*L* 2% *S*): No growth.
- Dextrose 1%—laevulose 1%—synthetic (*D* 1% *L* 1% *S*): Close resemblance to roots in *D* 2% *S*, but growth even slower.

(b) *In yeast culture solutions*

- Sucrose 2%—yeast 100 mg. (*S* 2% *Y* 100): Growth in length slow, laterals initiated often in considerable numbers, but slow growing or completely arrested, roots markedly thicker than in *S* 2% *S*, rapidly turning yellow and becoming brown in older cultures. The rate of growth in length varied according to sample of Difco yeast extract used. An old sample caused less retardation of growth in length than a new sample received during the course of the investigation.
- Sucrose 2%—yeast 10 mg. (*S* 2% *Y* 10): Similar to *S* 2% *Y* 100, but inhibition of growth in length less marked, some second-order laterals present.
- Sucrose 2%—yeast 2.5 mg. (*S* 2% *Y* 2.5): Growth in length intermediate between *S* 2% *S* and *S* 2% *Y* 100. Laterals produced in greater numbers per unit length than in *S* 2% *S*, well developed. Second-order laterals developed earlier than in *S* 2% *S*.
- Sucrose 2%—synthetic—yeast 2.5 mg. (*S* 2% *S* *Y* 2.5): Growth slightly more rapid than in *S* 2% *S*, appearance and growth habit similar, lateral development slightly superior.
- Sucrose 2%—synthetic—indoleacetic acid 0.01 p.p.m.: Growth in length very slow. Laterals initiated but arrested. Roots yellow at first but turning brown within 14 days of inoculation. Close resemblance to roots in *S* 2% *Y* 100, but inhibition of growth in length more marked.
- Dextrose 2%—yeast 100 mg. (*D* 2% *Y* 100): Growth slow, but superior to that in *D* 2% *S*, usually a few laterals developed, roots very pale yellow and intermediate in thickness between those in *D* 2% *S* and *S* 2% *S*, tips often curved and loops developed.
- Dextrose 2%—yeast 10 mg. (*D* 2% *Y* 10): Very closely resemble roots in *D* 2% *S*, laterals occasionally developed in old cultures.
- Dextrose 2%—synthetic—yeast 2.5 mg. (*D* 2% *S* *Y* 2.5): Very closely resemble roots in *D* 2% *S*, laterals occasionally developed in old cultures.

Dextrose 2%—synthetic—yeast 100 mg. (*D* 2% *S* *Y* 100): Growth as in *D* 2% *Y* 100.

(c) *In cultures employing purified sucrose and alcohol-soluble matter of sucrose*

Alcohol-precipitated sucrose (I) 2%—synthetic; alcohol-precipitated sucrose (II) 2%—synthetic; ether-extracted sucrose 2%—synthetic: Roots grown in these solutions were not distinguishable in increment rate, diameter, or growth habit from those in *S* 2% *S*.

Dextrose 2%+synthetic+concentrate of alcohol-soluble matter of sucrose (\equiv to 2% sucrose): Sucrose content of solution 7.5 mg. per 50 ml. Roots grew more rapidly in this solution than in any other dextrose solution and showed considerable branching. The roots, however, remained thin and translucent in appearance and the total growth was neither extensive nor prolonged.

2. *Growth increment and root diameters*

The growth increments and results of statistical analysis are shown for a number of culture solutions in Table I. The mean root diameters and results of statistical analysis for the solutions *S* 2% *S*; *S* 2% *Y* 100; *D* 2% *S*; and *D* 2% *Y* 100 are shown in Table II.

3. *Anatomy*

A detailed comparison has been made between roots grown in the four solutions *D* 2% *S*, *D* 2% *Y* 100, *S* 2% *S*, and *S* 2% *Y* 100 with the results shown in Table II. The quantities measured were selected as being those which seemed to give the best account of the structure of the root as a whole. The number of piliferous layer cells and the number of endodermal cells are related to the diameters of root and stele respectively. The number of cells in the exodermis (not counted) is in each case about half the number of piliferous layer cells. Partly because of the large numbers involved and partly because of the extreme difficulty in some instances of distinguishing between cells and intercellular spaces, the counting of cortical cells was found to be impracticable. The number of layers of such cells was therefore counted, taking advantage of the fairly regular arrangement of the cells in concentric circles. This, taken in conjunction with the cortex thickness measured from the outer wall of the endodermis to the inner wall of the exodermis, gives an indication of the effect of the various media on cell-size. Owing to the marked departure of many of the steles from a true cylindrical form, apparently as a result of secondary developments, measurements of stele diameter were not regarded as giving a satisfactory account of the primary condition of the stele. Instead, the width of the primary xylem was measured from protoxylem to protoxylem.

In studying the xylem it was necessary to establish a criterion by which to distinguish between primary and secondary vessels. We have shown elsewhere (Dormer and Street, 1948) that secondary xylem may be formed in considerable quantities in cultured roots. Many of the roots grown in sucrose

TABLE I
Growth Increments of Excised Tomato Roots (Best-of-All) grown in Various Culture Solutions

SERIES I	Culture solution.	Mean growth increment (mm./culture/day).	Significant at 1% level.
Sucrose 2%—synthetic (S 2% S)	.	5.7	$S\ 2\% S = S\ 2\% S\ Y\ 2.5, S\ 2\% Y\ 100, D\ 2\% Y\ 100$
Sucrose 2%—synthetic—yeast 2.5 mg. (S 2% S Y 2.5)	.	6.1	
Sucrose 2%—yeast 100 mg. (S 2% Y 100)	.	3.4	
Dextrose 2%—yeast 100 mg. (D 2% Y 100)	.	1.8	
SERIES II			
Sucrose 2%—synthetic (S 2% S)	.	11.8	$S\ 2\% S > S\ 4\% S = S\ 2\% Y\ 2.5 > D\ 2\% Y\ 100 > S\ 2\% Y\ 100 = D\ 2\% S$ $D\ 2\% S = D\ 2\% Y\ 10$ $S\ 2\% Y\ 100 > D\ 2\% Y\ 10$
Sucrose 4%—synthetic (S 4% S)	.	3.4	
Sucrose 2%—yeast 2.5 mg. (S 2% Y 2.5)	.	3.1	
Sucrose 2%—yeast 100 mg. (S 2% Y 100)	.	1.0	
Dextrose 2%—synthetic (D 2% S)	.	0.8	
Dextrose 2%—yeast 10 mg. (D 2% Y 10)	.	0.6	
Dextrose 2%—yeast 100 mg. (D 2% Y 100)	.	1.4	

TABLE II
Mean Root Diameters and Quantitative Anatomical Values for Excised Tomato Roots (Best-of-All) grown in Various Culture Solutions

Character and unit of measurement.	Mean values						Percentage increases (significant at the 1% level) resulting from the use of:			
	S 2 0' S. D 2 0' S. S 2 0' Y 100. D 2 0' Y 100.						Sucrose instead of dextrose		Yeast instead of the synthetic supplement	
							In yeast solutions.	In synthetic solutions.	In sucrose solutions.	In dextrose solutions.
Root diameter (microns)	370	160			640	240	167	131	73	50
Cortex thickness (microns)	108	32			165	44	271	232	52	37
Xylem width (microns)	116	62			120	61	95	87	—	—
Number of cells in piliferous layer	82.3	55.3			99.9	50.0	100	49	—	—
Number of layers of cortical cells	3.08	2.09			4.76	1.85	157	44	55	—
Number of endodermal cells	21.2	17.1			24.2	18.1	34	24	—	—
Number of primary xylem vessels	8.4	9.1			9.6	8.2	16	—	—	—

solutions contain vessels which, in view of their relationships with adjacent cells of the 'conjunctive parenchyma', cannot be regarded as primary. In many cases the status of a particular vessel cannot be certainly established. The procedure adopted is based on a study of those sections which contain no vessel which is clearly to be regarded as secondary. In such cases it is always found that the vessels are arranged in a single row, except sometimes at the protoxylem points where the vessels may be placed two or three abreast. It was therefore decided to count as secondary any vessel additional to this single row and attached to its middle portion.

The right-hand columns of Table II show those differences which are significant at the 1 per cent. level. Each difference is expressed as the percentage increase resulting from the use of sucrose instead of dextrose or of yeast extract instead of the synthetic vitamin supplement.

Transverse sections of selected roots from each solution are shown in the Text-figures, 3-6, all drawn to the same scale. The pericycle, phloem, and conjunctive parenchyma, which do not afford useful comparative features, are omitted. The cortical air spaces, the endodermal cells, and the secondary xylem vessels are marked in all figures.

No detailed comparison with roots of tomato plants grown in soil has yet been carried out, but it is clear that they show significant differences from the excised roots. In particular, the cultured roots are constantly diarch whereas, in tomato plants grown in soil, although the radicle is constantly diarch, the other roots may have any number of protoxylem groups up to five.

4. Changes in carbohydrate composition of the culture solutions

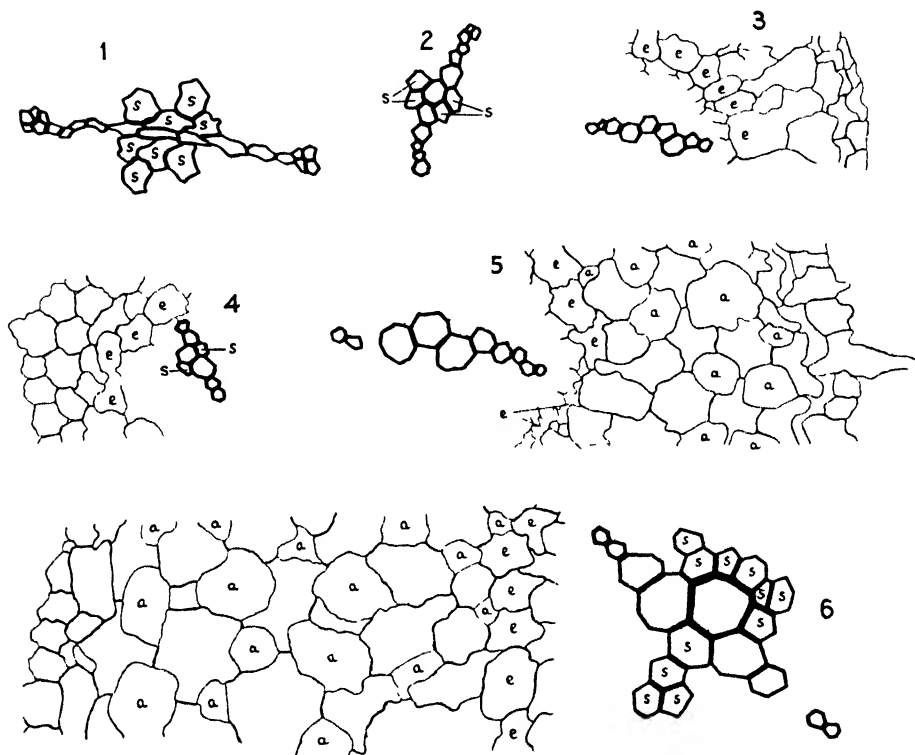
Determination of the reducing sugars in dextrose culture solutions after completion of growth of the roots (cultures 3-4 months old were used) revealed that the rate of dextrose utilization was so extremely slow that the change in dextrose content was only just detectable by the method of estimation used. Specimen results are shown in Table III.

TABLE III

Utilization of Dextrose by Excised Tomato Roots (Best-of-All) during Total Period of Growth

Culture solution.	Culture no.	mg. dextrose per ml. culture solution	
		Found.	Utilized.
<i>Dextrose 2 %—yeast 100 mg.</i>	{ Blank	19.50	—
	{ 12/103	19.21	0.29
	{ 12/104	19.30	0.20
<i>Dextrose 2 %—synthetic—yeast 2.5 mg.</i>	{ Blank	19.10	—
	{ 12/74	19.01	0.09
	{ 12/75	19.06	0.04
<i>Dextrose 1 %—synthetic—yeast 2.5 mg.</i>	{ Blank	9.47	—
	{ 12/82	9.32	0.15
	{ 12/83	9.31	0.16
	{ 12/84	9.34	0.13

The changes in carbohydrate composition occurring during the growth of roots in *sucrose 2%—synthetic*, *dextrose 1%—sucrose 1%—synthetic* and in *dextrose 0.5%—sucrose 0.5%—synthetic* solutions are shown in Table IV. The analytical values for sucrose, dextrose, and laevulose are here expressed as mg. $C_6H_{12}O_6$ per ml. culture solution, i.e. a solution containing 2 per cent. sucrose would be recorded as containing 21.05 mg. sucrose per ml.



FIGS. 1-6 (All $\times 220$.)

Transverse sections of xylem of radicles of etiolated seedlings 1. Grown in *sucrose 2%—inorganic* solution. 2. Grown in *dextrose 2%—inorganic* solution

Transverse sections of excised roots (phloem and pericycle omitted) 3. Grown in *dextrose 2%—yeast 100 mg.* solution. 4. Grown in *dextrose 2%—synthetic* solution. 5. Grown in *sucrose 2%—synthetic* solution. 6. Grown in *sucrose 2%—yeast 100 mg.* solution

a = air space, e = endodermal cell, s = xylem vessel considered to be secondary

Analysis of dextrose culture solutions taken after the roots had apparently ceased growth showed that only a minute quantity of the dextrose had been used (Table III). Similar analyses of solutions from old sucrose cultures showed that growth only ceases when the sucrose has completely disappeared. When roots are grown in sucrose+dextrose solutions growth continues as long as any sucrose is present and then ceases, although the dextrose concentration is then above that initially present (Table IV).

The utilization of sucrose is associated with accumulation of reducing sugars in the culture solution, and the analytical data (Table IV) point to the existence

TABLE IV
Changes in Carbohydrate Composition of Sucrose-containing Culture Solutions during Growth of Excised Tomato Roots (Best-of-All)

Culture solution.	Culture no.	mg. 'C ₆ H ₁₂ O ₆ ' per ml. culture solution.					A/B.	C/B.
		Sucrose	Dextrose.	Laevulose	Decrease in sucrose content	C ₆ H ₁₂ O ₆ utilized		
Sucrose 2%—synthetic	Blank	19.7	—	—	—	—	—	—
	12/108	17.02	0.99	1.36	2.68	0.35	0.50	0.13
	12/42	16.61	1.12	1.50	3.09	0.5	0.48	0.16
	12/18	15.5	1.35	1.95	4.2	0.9	0.46	0.21
	12/109	8.97	2.89	4.38	10.73	3.43	0.41	0.32
	12/5	5.0	3.61	7.2	14.7	3.9	0.49	0.25
	12/8	2.2	5.54	6.42	17.5	5.5	0.37	0.31
	12/4	nil	6.48	7.26	19.7	6.0	0.37	0.30
Dextrose 1%—sucrose 1%—synthetic	12/116	nil	6.3	8.8	19.7	4.6	0.45	0.23
	12/22	nil	7.0	8.7	19.7	4.0	0.44	0.20
						Mean values =	0.44	0.23
Dextrose 0.5%—sucrose 0.5%—synthetic	Blank	9.78	9.82	—	—	—	—	—
	11/136	4.2	11.3	2.74	5.8	1.34	0.49	0.24
	11/135	2.3	11.0	3.7	7.8	2.6	0.50	0.35
	11/134	1.3	11.4	4.3	8.48	2.6	0.50	0.30
Dextrose 0.5%—sucrose 0.5%—synthetic	Blank	4.25	4.95	—	—	—	—	—
	13/16	2.1	5.58	1.03	2.15	0.49	0.48	0.23
	13/12	0.96	5.74	1.68	3.29	0.82	0.51	0.25
	13/14	0.18	5.7	2.6	4.07	0.72	0.64	0.18
	13/18	0.11	5.8	2.4	4.14	0.89	0.58	0.21
	13/17	nil	5.82	2.46	4.25	0.92	0.58	0.22
	13/21	nil	6.0	2.56	4.25	0.64	0.60	0.15
	13/22	nil	5.74	2.32	4.25	1.14	0.54	0.27
	13/15	nil	6.1	2.4	4.25	0.70	0.56	0.16
	13/19	nil	6.11	2.41	4.25	0.68	0.56	0.16
						Mean values =	0.55	0.23

* Both dextrose solutions taken together.

of a quantitative relationship between the decrease in sucrose content and the carbohydrate assimilated, the ratio {mg. $C_6H_{12}O_6$ assimilated (C)/decrease in sucrose content (as mg. $C_6H_{12}O_6$) (B)} only varying within fairly narrow limits whether dextrose is present or not. This argues against an extracellular inversion of sucrose independent of hexose absorption and suggests rather the occurrence of a mechanism by which the breakdown of sucrose is linked with sugar absorption. The mean value of this ratio at 0.23, for both sucrose and dextrose+sucrose solutions, corresponds closely to the utilization of one hexose unit for each two molecules of sucrose degraded. Analysis of the accumulating reducing sugars into dextrose and laevulose shows that the mean value for the ratio {mg. laevulose accumulating (A)/decrease in sucrose content (as mg. $C_6H_{12}O_6$) (B)} stands at 0.44 for sucrose solutions and at 0.55 for sucrose+dextrose solutions. The greater part of the laevulose arising from the sucrose degradation is thus accumulated in the external medium and the sugar absorption is at the expense of the dextrose moiety.

Seedling Cultures

The root system of the seedling during its passage through the sand layer gives off fine laterals, but the radicle after entering the culture solution remains unbranched. Diameter measurements were made on unbranched sectors of 4 radicles from each culture solution (Table V). Unbranched root sectors

TABLE V

Mean Root Diameters and Quantitative Anatomical Values for Etiolated Seedling Radicles of Tomato (Best-of-All) growing in Sucrose—inorganic and Dextrose—inorganic Solutions

Character and unit of measurement.	Mean values		Percentage increases (significant at the 1% level) resulting from the use of sucrose solution instead of dextrose solution.
	Sucrose—Inorganic.	Dextrose—Inorganic.	
Root diameter (microns) .	491	230	113
Cortex thickness (microns) .	180	55	229
Xylem width (microns) .	156	67	133
Number of cells in piliferous layer	104	69	51*
Number of endodermal cells	25	19	32

* Significant at 5% level.

from other radicles were sectioned as described under anatomical technique. The results of the anatomical investigation are shown in Table V, in which a slightly less stringent standard of significance has had to be adopted for the count of piliferous layer cells, the difference between means in this instance being significant at the 5 per cent. level only. These roots are not illustrated completely, but their xylem groups are shown (Figs. 1 and 2) on the same scale as the other sections.

DISCUSSION

The present investigation commenced with a study of the growth of excised tomato roots in the four culture solutions: *sucrose 2 %—synthetic*; *sucrose 2 %—yeast 100 mg.*; *dextrose 2 %—synthetic*; *dextrose—yeast 100 mg.* The higher growth-rate and greater diameter of the sucrose as compared with the dextrose roots was immediately apparent. Superimposed upon this was a somewhat different effect of yeast as against the synthetic organic supplement according to the carbohydrate used. Whilst the yeast extract, at the comparatively high concentration of 100 mg. per litre, increased root diameter with both sucrose and dextrose, the effect on growth-rate was to cause a marked reduction in the presence of sucrose and by contrast a slight increase with dextrose. Further investigation of the phenomenon has involved anatomical study of excised roots from the four culture solutions listed above and of attached radicles growing into both sucrose and dextrose solutions; investigation of the rate and habit of growth of excised roots in a range of other culture solutions and study of the course of sugar utilization in dextrose, sucrose, and dextrose+ sucrose culture solutions.

Robbins and Schmidt (1938) found that the different sucrose and maltose samples which they used differed markedly in their growth effects on excised tomato roots, and as this was not related to the ash constituents of the sugar samples, it appeared that the presence or absence of minute traces of beneficial organic impurities might account for the conflicting results obtained by different workers studying the carbohydrate nutrition of excised roots. The presence of significant growth-promoting alcohol-soluble impurities in certain sucrose samples seemed probable from the earlier work of Hall, James, and Stuart (1933) on the growth of yeast and of Allison and Hoover (1934) on the growth of legume nodule bacteria. It was also to be expected that such impurities of natural origin would be likely to suffer destruction during the commercial preparation of dextrose. In our preliminary experiments the slight stimulation of growth in length resulting from the presence of yeast extract in dextrose solutions as compared with the retardation observed with sucrose suggested the possibility that the sucrose might contain a growth factor in stimulatory concentration which was absent or present in sub-optimal concentration in dextrose. We might then expect that if yeast also contained this factor the addition of yeast to sucrose could result in an excessive (inhibitory) concentration of the factor whilst addition of yeast to dextrose would provide the factor and lead to growth stimulation. This view was further supported by the observation that solution *S 2 % S Y 2.5* gave a higher growth increment value than *S 2 % S*, but that with solutions *S 2 % Y 10* and *S 2 % Y 100* inhibition occurred (Table I). Further, whilst *D 2 % S Y 2.5* had a growth increment value not significantly different from *D 2 % S*, both *D 2 % S Y 100* and *D 2 % Y 100* showed enhanced growth increments (Table I). Anatomical comparison of yeast and synthetic cultured roots shows that the increase in root diameter caused by the yeast

depends upon an inflation of the cortex caused by both increase in the number of cortical cells and in their size. Furthermore, the inhibition in growth in length resulting from yeast in presence of sucrose is associated with initiation of numerous laterals but arrest of their development. Examination of roots grown in solutions $S\ 2\%$ $Y\ 2.5$ and $S\ 2\%$ $Y\ 2.5$ shows that at this low concentration yeast enhances development of laterals, particularly those of the second order. These results suggested that the effect of yeast was partly to be explained in terms of its auxin content (Delarge, 1941; Burström, 1942). It is known that indole-acetic acid at concentrations below 10^{-11} g. per ml. causes slight stimulation, and at concentrations above this marked retardation of growth in length of excised tomato roots (White, 1943). Control roots grown in $S\ 2\%$ S containing 0.01 p.p.m. indole-acetic acid showed marked resemblance to our $S\ 2\%$ $Y\ 100$ roots. It was quite clear, however, that the difference between sucrose- and dextrose-grown roots could not be explained on the basis of a favourable auxin content in the sucrose, and that introduction of a favourable content of auxin into dextrose solutions did not lead to the production of roots similar to those grown in sucrose. In order, therefore, to test for the presence of a significant impurity in the sucrose, an attempt was made to separate both ether-soluble and alcohol-soluble impurities from the sucrose sample used and then to see if the procedure had decreased the growth activity of the sucrose or had yielded an 'impurity' fraction markedly stimulatory when added to dextrose solutions. Both the sucrose samples prepared by alcohol-precipitation and by ether-extraction were equal in growth activity to the initial sucrose sample. The 'impurity' concentrate when added to dextrose did result in some growth stimulation, but this was transitory and was probably due to the contamination with sucrose (7.5 mg. sucrose per 50-ml. culture solution were present). Furthermore, the roots remained typically dextrose roots in diameter and appearance. It was therefore concluded, in agreement with White (1940), that there was no evidence indicating the presence in the sucrose used of impurities markedly beneficial for the growth of excised tomato roots.

White (1940a) reported that excised tomato roots grown in dextrose solutions became brown and swollen, and he concluded (White, 1943) that the unsatisfactory behaviour of monosaccharides is probably due to the presence of injurious impurities. It is also reported that dextrose is relatively thermolabile and that sugar acids are produced by prolonged autoclaving (Smith, 1932; Thielman, 1938). Robbins and Schmidt (1938), however, recorded that their dextrose-grown tomato roots were more slender and translucent than the sucrose-grown roots and that swelling of the tips occurred less frequently and was less pronounced in dextrose than in sucrose. Their dextrose solutions were therefore quite free from toxic effects, although sterilized by autoclaving (15 lb. for 15 minutes). In the present investigation the roots in dextrose solutions, though extremely slow growing, apparently resembled in diameter and colour those of Robbins and Schmidt (1938) and were quite free from the toxic symptoms recorded by White (1940a). The pH values of the dextrose

solutions were found to be within 0.2 of the values for the corresponding sucrose solutions, indicating that no appreciable acid drift occurred in the dextrose solutions during autoclaving. The absence of toxic impurities in the dextrose was, however, most clearly demonstrated by the rapid growth of roots in solutions containing mixtures of sucrose and dextrose. It is therefore not possible to explain the poor growth of our excised tomato roots in dextrose solutions as due to the presence of toxic impurities or decomposition products in the dextrose.

The four culture solutions used in our initial experiments involve application of both sugars at 2 per cent. concentration so that they were comparable in carbon content but unequal in osmotic pressure. It was therefore possible that an osmotic effect might be involved. Roots in *sucrose 4%—synthetic* solution showed a lower growth increment value than in *sucrose 2%—synthetic*, but the general habit of growth and root diameter was similar in both solutions. Roots grown in dextrose—1% solutions very closely resembled those in dextrose—2% solutions in growth increment value, habit of growth, and root diameter. Whilst it is seen from these experiments that the osmotic value can have an effect on the growth increment value, this factor is clearly not sufficient to account for the differences observed between sucrose- and dextrose-grown roots.

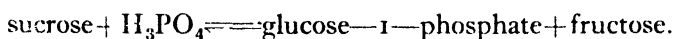
The anatomical evidence (Table II, Figs. 3–6) shows that the roots grown in sucrose solutions are larger and contain a greater number of cells than the corresponding dextrose roots. Whilst substitution of yeast extract for the synthetic organic supplement of White's medium leads merely to enlargement of the cortex, change in the carbon source alters the size and structure of every tissue of the root. In the sucrose roots active cell division in the conjunctive parenchyma leads to early and considerable development of secondary xylem vessels, and frequently intrusion of parenchyma cells into the primary xylem plate isolates protoxylem vessels (Figs. 5 and 6). In the dextrose roots secondary xylem is usually absent or, much less frequently, one or two secondary vessels are developed (Figs. 3 and 4). A comparison of transverse sections of seedling radicles growing into *sucrose—inorganic* and *dextrose—inorganic* solutions reveals a similar contrast in structure to that seen with the excised roots. Not only are the differences in the same sense, but they are of similar magnitude (Table V). The radicles are, however, in some respects better developed than the excised roots as instanced by the considerable amount of secondary xylem often developed in dextrose radicles (Fig. 2). The sucrose radicles (Fig. 1) show that the primary xylem vessels fail to lignify and become compressed and distorted by the well-developed secondary vessels. This is probably due to a very early development of secondary xylem in the sucrose-grown seedlings.

This leads to a consideration of the evidence that the superiority of sucrose over dextrose as a carbon source for excised tomato roots is due to the fact that sucrose is more readily utilized. In *laevulose 2%—synthetic* solution no growth was recorded, while *dextrose 1%—laevulose 1%—synthetic* solution

was slightly inferior to *dextrose* 2 %—*synthetic* solution. The sugar analyses also show that dextrose is utilized extremely slowly and that growth ceases with the initial dextrose concentration little changed (Table III), whereas in sucrose solutions growth only ceases when the sucrose has been completely broken down (Table IV). Furthermore, the evidence indicates that free dextrose is not utilized at all when sucrose is present. These results seem to show that excised tomato roots are able to utilize freely neither dextrose, laevulose, nor an equimolecular mixture of the two.

A study of the changes in carbohydrate composition occurring in the culture solution (Table IV) as a result of the growth of excised tomato roots supplied with sucrose as carbon source points to the existence of a mechanism involving a breakdown of the sucrose molecule into its hexose units coincident with the absorption of one 'dextrose' unit for each two molecules of sugar degraded. This characteristic mechanism of sucrose utilization is little affected by the initial presence or absence of free dextrose.

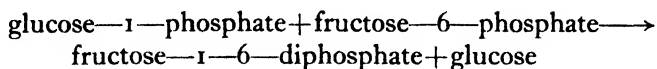
Verzár (1935) has shown that the absorption of sugars by the intestinal wall of mammals is specific for certain sugars and is limited in rate, and Lundsgaard (1933) has found that the absorption of dextrose can be depressed, in a fully reversible manner, by phlorizin, which is an inhibitor of phosphorylation. Gomori (1939) and Kritzler and Gutman (1941) have demonstrated that the proximal tubules of the kidney which are responsible for sugar reabsorption are particularly rich in phosphatase and that it is concentrated at the luminal border of the epithelium. Hober (1946), in a comprehensive review of the evidence, has visualized the possibility that sugar absorption by the cell involves, at the pH of the cell surface, phosphorylation and entry followed, at the pH of the cytoplasm, by dephosphorylation with liberation into the cell of free sugar which has shifted across the boundary as a hexose phosphate. Doudoroff (1940) has shown that *Pseudomonas saccharophila* grows more rapidly and respire more actively when supplied with sucrose than when supplied with dextrose, and is almost incapable of utilizing laevulose. Dried cells of this organism ground to a fine suspension in water rapidly esterify inorganic phosphate at pH 6.3–7.0 in the presence of sucrose (Doudoroff, Kaplan, and Hassid, 1943) and the over-all phosphorolysis can be represented thus:



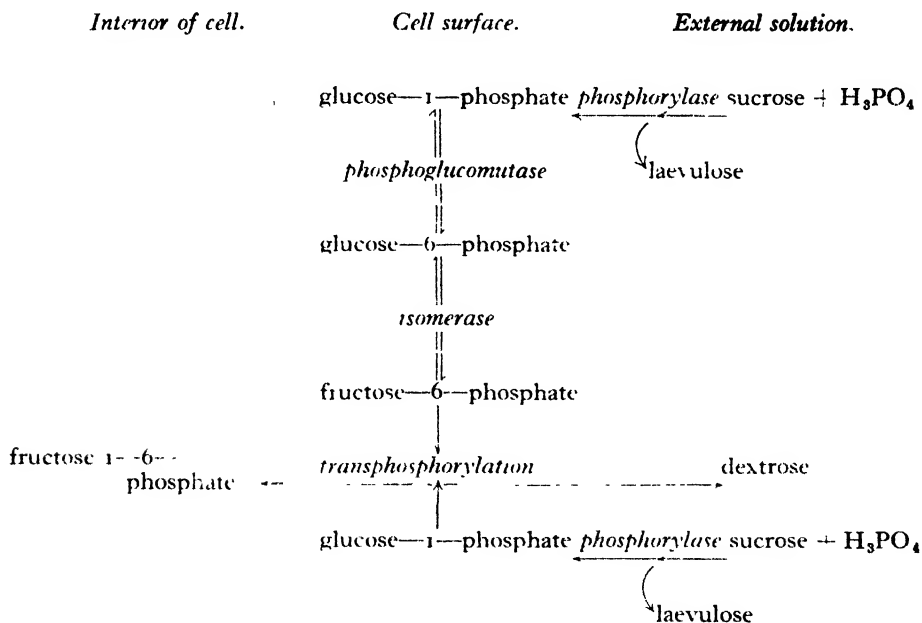
Neither the preparation of dried cells nor the actively growing organism could esterify inorganic phosphate when supplied with dextrose as the sole carbon source. The preparation of dried cells contained a hydrolytic system active against sucrose and competing with the phosphorylase. In a later paper Doudoroff (1943) obtained the phosphorylase system almost free from hydrolytic activity and demonstrated that it was specific for sucrose and that no co-enzyme appeared to be necessary. The equilibrium constant

$$K = \frac{(\text{sucrose})(\text{phosphate})}{(\text{glucose—1—phosphate})(\text{fructose})}$$

was approximately determined at 30° C. and the values obtained were 0.05 (pH 6.6) and 0.09 (pH 5.8). The equilibrium, therefore, corresponds to almost complete phosphorolysis of sucrose. Doudoroff considered that the inability of *P. saccharophila* to utilize dextrose was a consequence of the specificity of the phosphorylase for sucrose and postulated that free hexose was unable to pass the cell membrane. Lipmann (1941), in considering the efficiency of energy utilization by muscle supplied with glycogen, has suggested that the following transphosphorylation may take place, although the enzyme system involved is as yet unknown:



With these considerations as a background and as a basis for further investigation the following hypothesis of the mechanism of sucrose absorption by excised tomato roots is tentatively advanced:



If a mechanism of this type is operative, and if the entry of carbohydrate into the cytoplasm involves the formation of a hexose phosphate, it is to be expected that the rate of sucrose utilization will be affected by the pH and the inorganic phosphate content of the external solution and that more rapid utilization of dextrose may be induced by supplying a glucose phosphate or by providing a phosphate donor such as adenosine polyphosphate together with the free dextrose. Investigations along these lines are now proceeding. This hypothesis also suggests an explanation for the otherwise anomalous

stimulation of dextrose roots by a high concentration of yeast extract, since yeast is the richest known source of adenosine polyphosphate containing 80 mg. of polyphosphate-P per 100 g. (Lipmann, 1941).

SUMMARY

1. By comparing excised tomato roots grown in different culture solutions it is found that roots supplied with sucrose are thicker, quicker growing, and more abundantly branched than those grown on dextrose, and show greater anatomical differentiation.

2. A similar contrast in diameter and anatomy is observed between the radicles of etiolated seedlings supplied with the two sugars.

3. It is shown that these differences cannot be explained in terms of impurities in the sugars nor as an effect of the osmotic pressures of the culture solutions.

4. From the changes which occur in the carbohydrate composition of the culture solutions during growth it is concluded that the roots are able to utilize sucrose at a much greater rate than either dextrose, laevulose, or an equimolecular mixture of the two.

5. A possible mechanism of sucrose utilization is suggested, involving a specific phosphorylation of sucrose.

6. Both sucrose and dextrose solutions containing yeast extract yield thicker roots than the corresponding solutions containing a synthetic organic supplement. Yeast also has a marked effect on the rate of elongation.

7. The thickening due to yeast extract involves only the cortical tissue, whereas that due to sucrose involves changes in all the tissues.

8. The effects of yeast extract are interpreted as effects of the growth hormone and polyphosphate-P which it contains.

We wish to express our sincere thanks to Mrs. K. J. Dormer for preparing Figures 1-6, and to the Research Committee of the University of Nottingham for a special research grant.

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Studies in the Physiology of Obligate Parasitism

II. The Behaviour of the Germ-tubes of Certain Rusts in Contact with Various Membranes

BY

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(School of Agriculture, Cambridge)

With six Figures in the Text

INTRODUCTION

IT has long been known that the tip of a hypha may swell on coming into contact with a leaf. Such swellings, called appressoria, have usually been considered as the reaction to a contact stimulus. Owing perhaps to the difficulty of experimental work, little has been done except by observation.

Swellings at the apex of germ-tubes of rust fungi were first observed by the Tulasne brothers (1847). Some have thought that such swellings were due to abnormal conditions of germination, but Butler (1918) figured such a case and called it an appressorium. Ezekiel (1930) considered these swellings were incipient teleutospores, but admitted that proof was lacking. Robinson (1919) noticed that they occurred on contact with leaf-surfaces; Butler also obtained them in contact with glass coverslips (correspondence). Allen (1926) directed attention to possible contact reactions in her description from cytological preparations of haustorial formation in *Puccinia triticina*. She wrote: 'When the tip of a hypha strikes against a host cell, and its growth is forcibly checked for the moment, the changes preparatory to haustorium formation set in.' Although Miss Allen was referring to the hypha in the leaf, it seems possible that such reactions might also take place when a germ-tube comes into contact with the guard-cells of a stoma.

The morphology of the appressorium and substomatal vesicle and the number and direction of growth of the infecting hyphae arising from the vesicle have been described by Pole Evans (1907) in certain rusts. In particular the rather oval- to pear-shaped vesicles of *P. glumarum* and *P. triticina* contrast with the elongated bifurcate vesicle of *P. graminis*.

In Paper I (Dickinson, 1949) the positive hydrotropism, negative thigmotropism, and diathigmotropism, i.e. growth along a membrane of the germ-tubes of certain rusts and mildews, were described. Their direction of growth on coming into contact with a membrane was shown to be away from or along the surface according to the condition of the membrane. In this paper the form of growth of the germ-tubes and infection hyphae of three rusts when in contact with certain membranes and mesophyll cells will be described.

The three rusts are *P. triticina* Erikss., *P. graminis* Pers., and *P. glumarum* (Schm.), Erikss. and Henn.

MATERIALS AND METHODS

Full details of the methods evolved for the making of collodion, gelatin, calcium pectate, and other membranes will be given elsewhere, together with the technique involved in the cytological examination of fungal cultures grown on such membranes. It will suffice to say that in making collodion membranes the required amount of collodion solution was poured on to a clean level glass sheet of known area and allowed to dry. After scratching circles of 1 in. diam. in the dry collodion, the separate membranes were floated off on water and mounted on metal rings. Such mounted membranes formed the partition in the double-chambered, Van Tieghem drop cell (Dickinson, 1949). The spores under investigation were germinated on the upper surface of the membranes, while the liquid below the membranes was always distilled water. The spores were put on to the membranes from a fine camel-hair brush. The brush holding the spores was tapped gently when about 2–3 in. above a membrane, so causing the spores to fall on to it. Care was taken to put only a few well-separated spores on each membrane, and as a rule no germ-tube came into contact with any other.

The cytological preparations of collodion membrane cultures were made by modification of a method previously described (Dickinson, 1927). The membrane was first exposed to the vapour of Fleming's fixative for 5 minutes, and then inverted on microscope slides smeared with Mayer's egg-albumen fixative. The germ-tubes were fixed to the microscope slide with a drop of glacial acetic acid, which was immediately washed off by flooding the preparation with 70/30 : alcohol/ether mixture. The slide was rapidly transferred to absolute alcohol through small changes of concentration and left there for an hour. After this hardening bath, normal staining with Heidenheim's haematoxylin was carried out and followed by counter-staining with light green. For comparative purposes Feulgen's nuclear reaction was used.

EXPERIMENTS WITH PARAFFIN-WAX COLLODION MEMBRANES

In Paper I of these studies the duration of the drying time of the membranes and the alcohol concentration of the soaking liquid were varied. In the experiments to be described at first a layer of wax was deposited from an ether solution on the dried collodion on the glass sheet. Later the wax was incorporated in the collodion solution, which then consisted of 0.4 per cent. collodion, 0.05 per cent. paraffin wax in 9/91 : alcohol/ether. The liquid below the membrane was distilled water.

The germ-tubes of *P. triticina*, when not in contact with a membrane, were unbranched (Fig. 1, *a*) except sometimes towards their apices, when the 'stagshorn effect' (Fig. 1, *b*), as seen by Sappin Trouffy (1896) and others, was observed. When branches were formed, they were usually fairly short and few in number (Fig. 1, *b*). Only occasionally were very short or knobbed

branches produced. The length of the germ-tube was fairly regular, about $800\ \mu$. Ezekiel (1930) reported a length of germ-tube in *P. graminis* of rather less than $1,000\ \mu$.

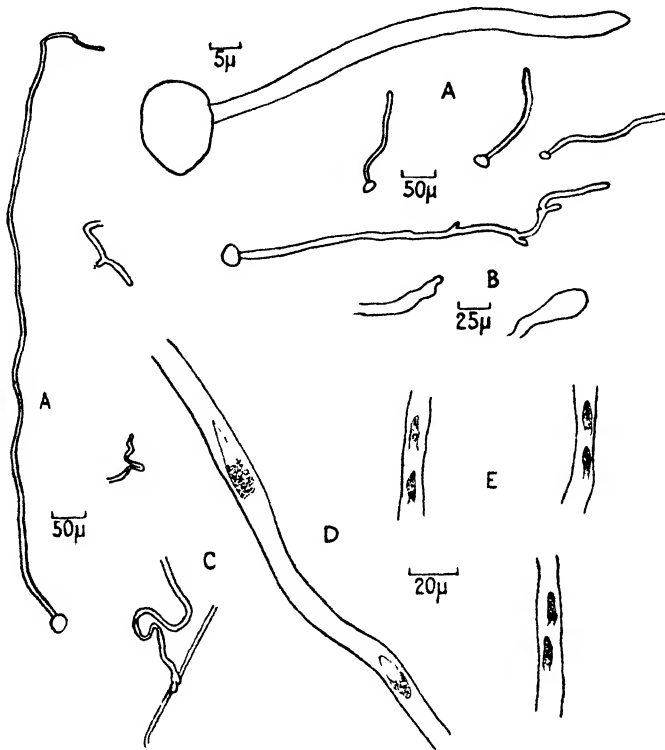


FIG. 1 The germ-tubes of *P. tritici* grown on water, 1.5% agar or 5% gelatin. *a*, Unbranched germ-tubes. *b*, Slight branching in germ-tubes, the 'stagshorn' effect. *c*, slight swelling developed when one germ-tube has come in contact with another. *d*, Two well-stained nuclei in a germ-tube. The complete nucleus can be seen. *e*, Pairs of nuclei in germ-tubes, showing the 'tail' effect due to light staining. The complete nuclear envelope cannot be seen.

All drawings in this and later figures have been made with the aid of a camera lucida, using a Spencer Lens microscope and 2/3rd, 1/6th, and 1/12th oil-immersion objectives and 6 \times , 10 \times , and 15 \times oculars. Except in Fig. 6, drawings in outline only are made from living material. Cytological preparations have been stained with Heidenheim's haematoxylin and light green. The preparations illustrated in Fig. 6 were stained with cotton blue dissolved in lactophenol and mounted in lactophenol and glycerine.

The habit of growth of the germ-tubes when in contact with a dry-surface membrane, such as paraffin-wax (congealing-point 36°C.) collodion, was characteristic; the germ-tubes were continually putting out side branches, none of which grew to any considerable length (Fig. 2, *a*, *b*). Later their diameter was reduced, no branches being formed, and growth was in a straight line (Fig. 2, *a*). Usually one or more patches of an orange-red colour were present in this straight hypha at the end of the germ-tube's growth, or less often in the branched part of the germ-tube. The branches were formed by

new growing-points continually arising in the penultimate part of the germ-tube, carrying on the growth for a short length and then giving place to a later-formed growing-point (Fig. 3, *a*). Shortly before the formation of a new growing-point, elongation stopped. Not all the daughter growing-points grew

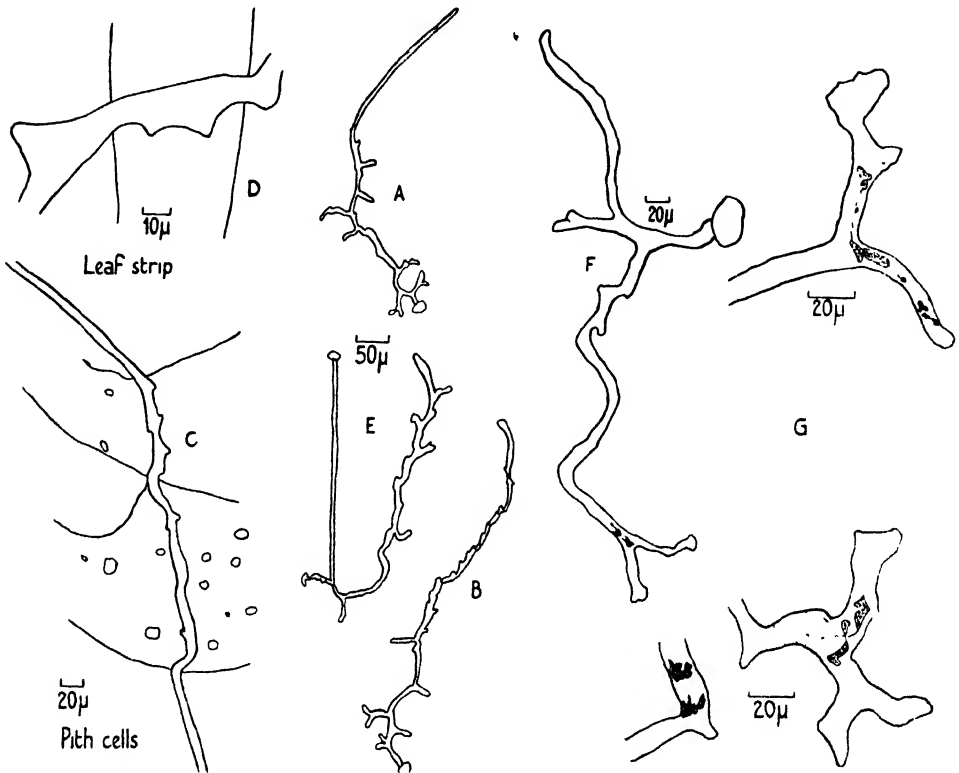


FIG. 2. The germ-tubes of *P. tritici*, those lettered *a*, *b*, *e*, *f*, and *g* being grown on paraffin-wax (c.pt. 36° C.) collodion membranes. *a*, Showing side branches, the 'branched' form of growth, and towards the end of growth a characteristic straight unbranched hypha has been formed. *b*, Showing an early 'branched' and later 'gnarled' form of growth. *c*, Germ-tube growing amongst pith cells. The central portion of the hyphal length is in contact with the pith cells and shows the formation of daughter growing-points. At either end the germ-tube did not come into contact with the pith cell-walls and has no daughter growing-points. *d*, Germ-tube growing among the mesophyll cells of a leaf. *e*, The germ-tube has at first grown in the air and is unbranched; later, having come into contact with the membrane, it has a mixture of 'branched' and 'gnarled' growth forms. *f*, A pair of nuclei, somewhat distorted in shape, towards the apex of a germ-tube, showing a 'branched' form of growth. *g*, Nuclear material of distorted shape, or with an enlarged chromatin network, or split up into a number of pieces.

even for a short length, but the continual formation of such growing-points gave a knobbed appearance to the hyphae (Fig. 3, *b*). This type of growth is called 'gnarling'. When the daughter growing-points grew for a short length, then the appearance was of a hypha with numerous side branches and the fully grown germ-tube had a jagged outline (Figs. 2, *a*; 3, *c*). This type of growth is called 'branching'. This repeated formation of daughter growing-

points was similar to the behaviour of the hyphae in the leaf described by Allen (1926). The two forms of germ-tube corresponded to the searching and clasping hyphae described by Marshall Ward in the leaf mycelium of *P. glumarum* (1904). Both forms of growth were also seen in different parts of the same germ-tube (Fig. 2, *b*). That the formation of daughter growing-points was a reaction to a contact stimulus was further demonstrated by the behaviour

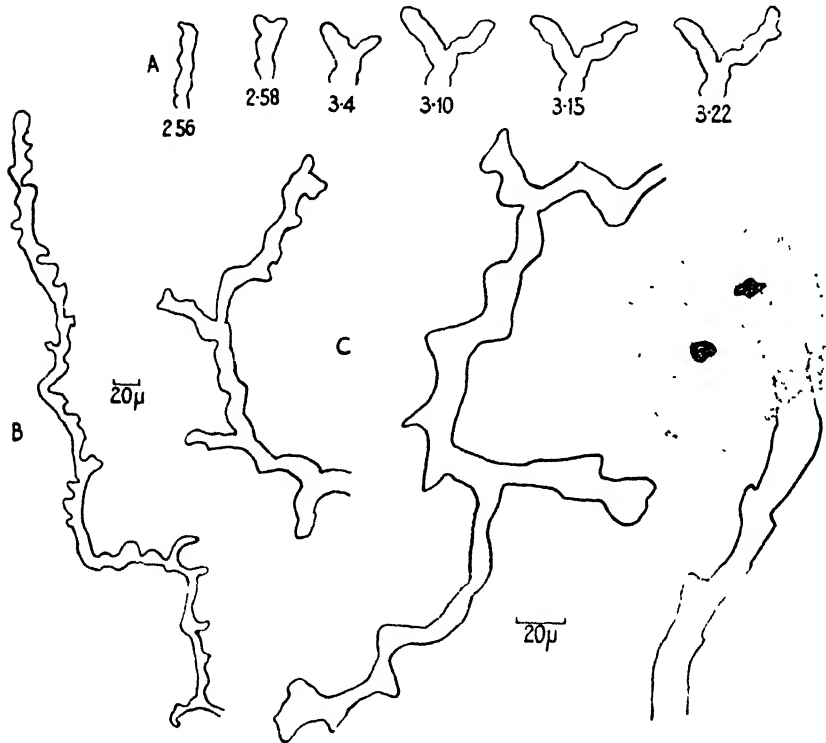


FIG. 3 The germ-tubes of *P. tritici* grown on paraffin-wax (c.pt. 36° C.) collodion membranes. *a*, A series of drawings made of a growing germ-tube to show the development of daughter growing-points. The time is shown in hours and minutes. *b*, The 'gnarled' form of growth. *c*, The 'branched' form of growth. *d*, A 'burst' in a germ-tube towards its apex, but slightly to one side. The circular wet patch of gelatinous material contains two bodies, stained by the Feulgen nuclear reaction, which are probably the nuclei.

of germ-tubes growing among pith cells (Fig. 2, *c*). So long as the hyphae were in contact with the wall of a pith cell, branching and the formation of new growing-points took place. As soon as a gap in the cell-wall was reached, no new growing-points were formed and the appearance was that of a straight unbranched hypha. It must be emphasized that, whether in contact with a membrane or not, only one growing-point was developed at a time; true branching never occurred. A similar habit of growth was seen when the germ-tubes were in contact with the mesophyll cells of a wheat leaf (Fig. 2, *d*), or with other hyphae (Fig. 1, *c*). The behaviour of *P. graminis* was similar,

though the number of daughter growing-points was less and the germ-tubes had a more regular outline. Usually their growth was undulating (Fig. 5, *c*), while the side branches were longer than in *P. triticina* (Fig. 5, *d*). The straight hypha at the end of the germ-tube's growth, described in *P. triticina*, was also seen. The patches of colour were more brownish-red in *P. graminis* in contrast to the orange-red in *P. triticina*.

Striking differences were seen in the cytological preparations of both species according to whether the germ-tubes were or were not grown in contact with a membrane. In almost all cases when the germ-tube was not in contact with a membrane, the nuclei were two in number, close together, and quite normal in appearance (Fig. 1, *d*). Except in the stagshorn form of hypha the nuclei were oval with a large vacuole. Most of the chromatic material was aggregated at one end (Fig. 1, *d*). In lightly stained preparations only this aggregation was visible and appeared to have two 'tails' standing out from its base (Fig. 1, *e*). Deeper staining showed that these 'tails' were part of the envelope of the large nuclear vacuole. In the stagshorn form of hypha the appearance of the nuclei was similar to that in hyphae grown in contact with membranes, though the distortion of the chromatic material was less pronounced in the former.

When the hyphae were in contact with paraffin-wax (c.pt. 36° C.) collodion membranes the form of the nucleus was variable (Fig. 2, *f, g*). One to four, sometimes more, pieces of deeply staining material were visible. The chromatic network was often greatly enlarged and distorted, though no clear and distinct nucleus was visible (Fig. 2, *g*). The distances between the pieces of chromatin material and between the two parent nuclei were variable, though the latter could not always be identified with certainty. The larger pieces of chromatin were usually found in the more normal-looking branches. The occurrence of such chromatic pieces, together with the distorted nuclear network, suggested strongly that nuclear division had been initiated, but under such conditions had not been completed. The orange or brown-red patches, seen in living material, had lost their colour in the cytological preparations, but were stained deeply with Heidenheim's haematoxylin. In such areas there was a massing of the cell contents, and the vacuoles had disappeared. This orange-red colour was similar to that seen in germ-tubes grown under water, or that which developed when they were dried quickly.

There was another phenomenon apparently connected with these attempts at branching and the initiation of nuclear division in both *P. triticina* and *P. graminis*. Towards the apex of the germ-tube the hypha burst and its contents shot out, forming a semicircular wet patch at its side. Sometimes this burst occurred at the apex, and then the wet patch was circular (Fig. 3, *d*). Examination of these ruptured hyphae and the extruded material showed two somewhat round bodies in a slightly granular gelatinous matrix. These two bodies, since they stained with both haematoxylin and the Feulgen nuclear reaction, were thought to be the nuclei. Generally the hyphae became swollen before bursting, their diameters being often as many as three times the normal.

These bursts were always preceded by a reddening of the contents in the region where the break was to take place; after the burst the orange or brown-red colour disappeared at once.

EXPERIMENTS WITH CELL-WALL PARAFFIN-WAX (c.pt. 36° C.) COLLODION MEMBRANES

Following the conclusion that initiation of nuclear division was part of a contact reaction, it was decided to add to the membrane some cell-wall material from the host leaf. The first seed leaves of a susceptible *Triticum vulgare* wheat were cut up and ground in a pestle and mortar with a little water. This ground material was thoroughly washed in tap-water and the solids retained on bolting silk. This solid material, pale cream in colour, was suspended in distilled water and drops of the suspension were placed on wax collodion membranes on glass. After the water had evaporated there was left on the collodion a thin layer of cell-wall debris. Examination showed that this was composed of collapsed cells without contents and also fragments of cell-wall. Further, this layer was not more than one collapsed cell thick. The membranes were floated off on water and mounted as usual. Similar membranes were made from the leaves of oats and barley, from the roots of all three cereals, from lawn-grass leaves, and from leaves of clover. All the results were similar and only those with wheat leaves will be described.

The diameters of the germ-tubes of *P. tritici* were rather larger on contact with these membranes than with the plain wax collodion membranes. Sometimes a succession of growing-points were formed and short branches were produced, but usually the germ-tube had an irregular outline with no branches. Patches of orange-red appeared, at first two in number, but later a limited spreading of the coloured granules took place. Sooner or later the diameter of the germ-tube at the growing-point increased considerably, and the growth in length ceased soon afterwards. The result was an irregularly oval mass at the apex of the germ-tube (Fig. 4, a). This oval mass will be called for the moment the 'basal swelling'. Less often an older part of the germ-tube grew in diameter and became a basal swelling. Only one basal swelling has been seen in each germ-tube. The whole of the protoplasm moved into this swelling and became a deep orange-red, while the rest of the germ-tube was now quite colourless. These basal swellings were firmly affixed to the membrane, and from them grew later a short stiff stalk bearing a pear-shaped body resembling a spore (Fig. 4, a, 3). On thin membranes these 'spore-like' bodies were usually produced below the membrane after its penetration by the stalk, but penetration did not occur on the thicker membranes. Occasionally basal swellings have been observed in germ-tubes growing away from the membrane. In such germ-tubes the swellings were always formed at the point of contact with one or more other germ-tubes and never produced spore-like bodies.

The stiff and elastic quality of the stalk and the firm adhesion of the basal swelling on the membrane were ascertained when attempts were made to

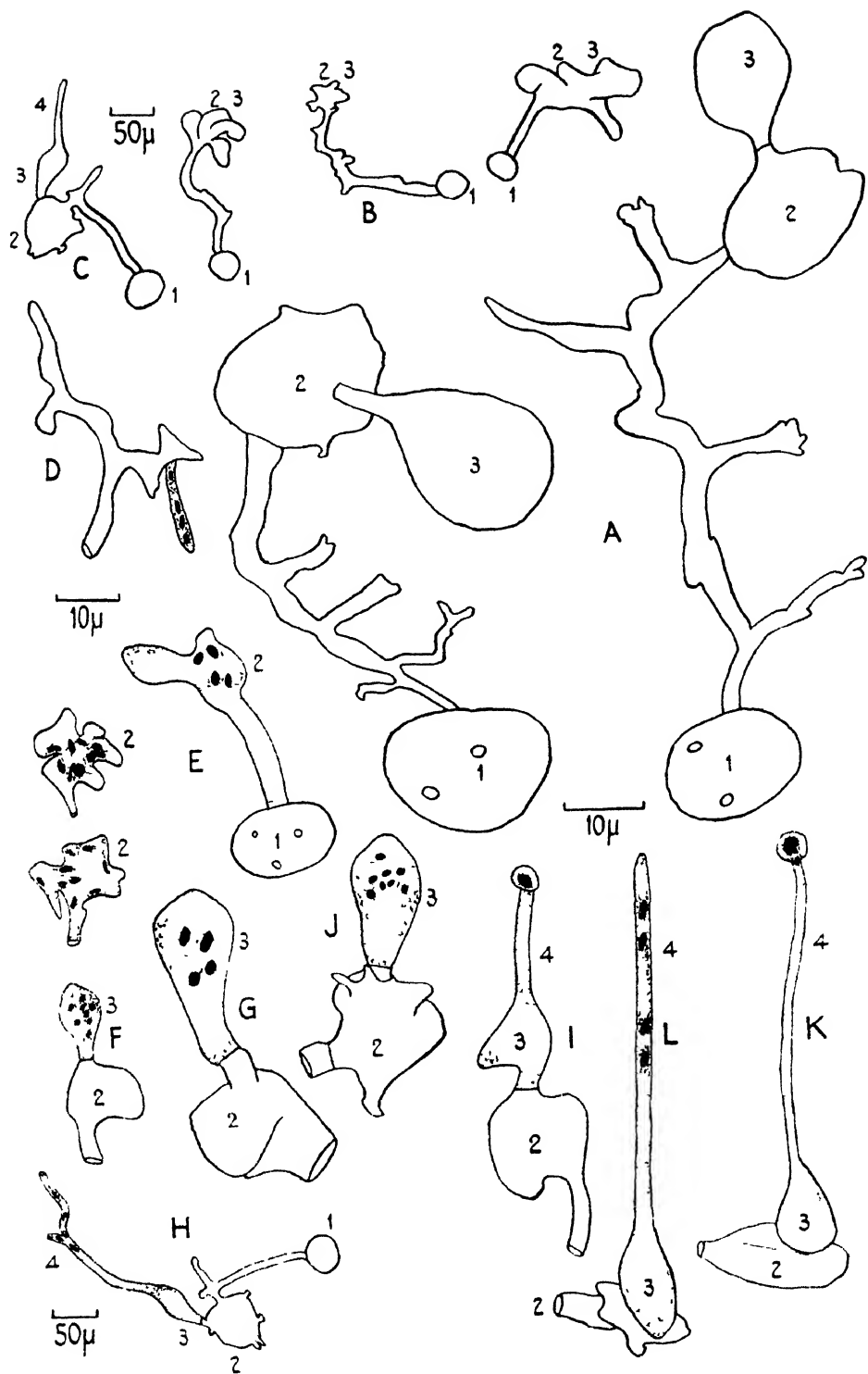


FIG. 4 (see opposite)

move them with an Isolator needle (Dickinson, 1933). This close adhesion was not due to the presence of a mucilaginous coat at the tip of the germ-tube. Repeated efforts to demonstrate its presence with indian ink have failed completely. The spore-like bodies had a warty skin and were rather similar to uredospores, except for the absence of germ pores, the rather more pear-like shape, and the slender base. The orange-red contents of the basal swelling migrated into the spore-like body, and a septum was then formed across the stalk. Later a short straight hypha developed from the spore-like body (Fig. 4, *k*, *l*), and usually grew away from the cell-wall material on which the parent body was formed. This hypha sometimes contained orange-red patches, but such coloration was not as characteristic as with the basal swellings and the spore-like bodies. If present, the colour always disappeared as soon as the hypha had grown to any length. These straight hyphae were reminiscent of the last stages of growth of germ-tubes grown in contact with ordinary wax collodion membranes. Sometimes there were signs of more than one hypha being produced, but such additional hyphae never developed to any extent. Less frequently on these, but commonly on some other membranes, a ball-like head was formed at the end of the straight hypha, which was then comparatively short (Fig. 4, *k*). The whole hypha had the appearance of a 'drumstick'. Such ball-like heads have also been seen to be formed directly by germ-tubes (Fig. 5, *a*, *e*). These 'drumsticks' were filled with protoplasm, but rarely had any colour except in their stalks. No further development has been observed.

When the uredospores of other rust species were tested, the basal swelling was found to vary slightly in form and size while the spore-like body took on a characteristic shape according to the species. In *P. graminis* it was cylindrical with the longer axis parallel to the membrane and a hypha was usually produced from each end. The first growth of these hyphae was parallel to the membrane, but later they grew away (Fig. 5, *k*). Their colour was more of a brown than an orange-red. In *P. glumarum* the basal swelling was rounded rather than pear-shaped as in *P. triticina*; and one straight hypha

FIG. 4. Drawings are of *P. triticina* germ-tubes. '1' indicates the parent uredospore, '2' the appressorium; '3' the substomatal vesicle, '4' the infection hypha. *a*, Normal substomatal vesicle formation. Right specimen grown on a cell-wall collodion membrane. Left specimen on a protein collodion membrane. *b*, Abnormal appressorial substomatal vesicle formation. Grown on paraffin-wax collodion membranes. *c*, Normal appressorium, substomatal vesicle, and infection hypha. Grown on a paraffin-wax collodion membrane. *d*, Abnormal appressorium producing an apparently normal infection hypha with 4 nuclei. Grown on a protein collodion membrane. *e*, Abnormal appressorial substomatal vesicles containing varying numbers of nuclei. Grown on a cell-wall collodion membrane. *f*, Normal appressoria and substomatal vesicles containing 8 nuclei. Grown on a cell-wall collodion membrane. *g*, *j*, Normal appressorium and substomatal vesicle containing 4 or 8 nuclei. Grown on a paraffin-wax collodion membrane. *h*, Normal appressorium, substomatal vesicle, and infection hypha. The latter had branched and has 2 nuclei in each cell. Grown on a cell-wall collodion membrane. *i*, *k*, Substomatal vesicles with infection hyphae, at whose apices a 'drumstick' has been formed, containing a single nucleus. Grown on a cell-wall collodion membrane. *l*, Substomatal vesicle and unbranched infection hypha containing 4 nuclei. Grown on a paraffin-wax collodion membrane.

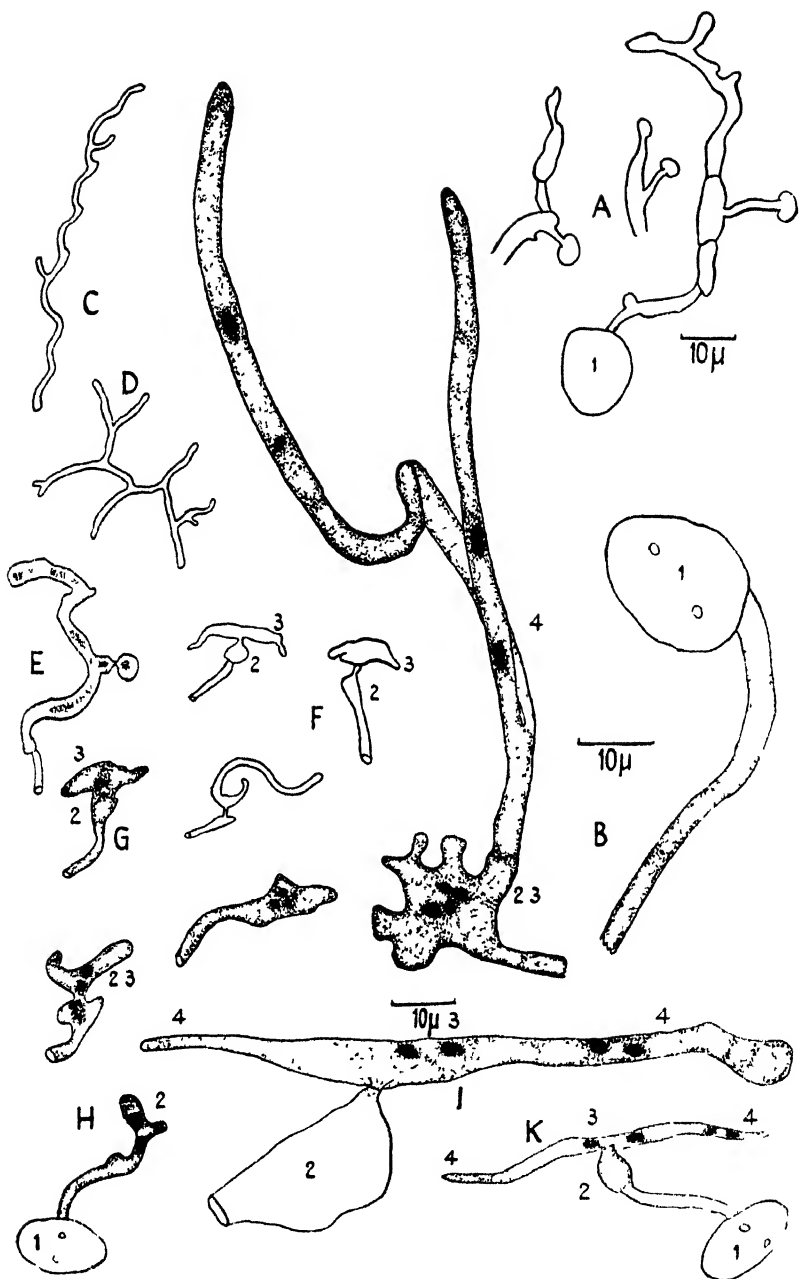


FIG. 5 '1' indicates uredospore, '2' appressorium; '3' substomatal vesicle; '4' infection hypha. Drawings: *a, b* are of *P. tritici*; *c-k* of *P. graminis*. Specimens drawn in *a, e* were grown on a paraffin-wax, formalized gelatin membrane; *b, g, h*, on a cell-wall collodion membrane; *c, d, f* on a paraffin-wax collodion membrane; *k*, on a protein collodion membrane. *a*, Germ-tubes showing 'balling'. *b*, 'bnormal appressorial substomatal vesicle producing a branched infection hypha with no septum. *c*, Germ-tube, an undulating form of growth. *d*, Germ-tube showing 'branching'. *e*, Germ-tube showing 'balling'. *f*, Appressoria and substomatal vesicles. *g*, Young substomatal vesicle and appressorium. *h*, Abnormal appressorial substomatal vesicles. *i, k*, Appressoria and substomatal vesicles producing infection hyphae.

was produced from the spore-like body. The colour was yellow to yellow-orange. Comparison (see Table) was made with the appearance of appressoria and substomatal vesicles in naturally infected leaves described and illustrated by Pole Evans (1907). This showed that the so-called basal swelling had the same form of the appressorium on the stomata of the leaf, the uredospore-like body had the well-defined form of the substomatal vesicle; while the straight hyphae were similar in number and appearance to the infecting hyphae. The ball-like head or drumstick was somewhat similar to a haustorium.

Neither in Pole Evans's (1907) nor in Allen's (1926) description of the appressoria and substomatal vesicles, nor in any other account yet found in the literature, has reference been made to the presence of an orange-red, brown-red, or yellow coloration. When living substomatal vesicles formed on the inside of the stomata of wheat leaves were examined, it was found that the substomatal vesicle in *P. tritici* was orange-red in colour, that of *P. graminis* brown-red, and that of *P. glumarum* yellow. This examination was made by putting spores on the under side of leaves and after 48 hours stripping off the under epidermis. Microscopic examination showed that the appressoria were sometimes coloured and that the substomatal vesicles were always coloured (see Table). This discovery led to the conclusion that the so-called 'basal swelling' is the appressorium, the uredospore-like body is the substomatal vesicle, and the straight hyphae are the infecting hyphae. Finally it was concluded that on the appropriate membrane, in this case made of cell-wall paraffin-wax collodion, the whole phenomena of entrance characteristic of these rust species could be reproduced. Variation from the normal appearance of the appressorium and substomatal vesicle was found less frequently on cell-wall collodion membranes than on some other membranes. Such variation included considerable irregularity in the form of the appressorium (Figs. 4, *b*; 5, *f*), and failure to develop a substomatal vesicle (Figs. 4, *e*; 5, *h*). The growth of only one infecting hypha was common in *P. graminis* with distilled water below the membrane. When sucrose was used, usually both infecting hyphae grew to a considerable length. Infecting hyphae have been found growing from very irregular appressoria, without substomatal vesicle formation (Fig. 4, *d*). Once in *P. tritici* a branched hypha without a septum was grown from a rather irregular appressorium (Fig. 5, *b*). The formation of 'drumsticks' was observed sometimes without the formation of substomatal vesicles (Fig. 5, *a*, *e*) in both *P. tritici* and *P. graminis*.

Cytological examination of the germ-tubes forming appressoria and substomatal vesicles which were grown on these and other membranes as well as on leaves confirmed the description given by Allen (1926). After the aggregation of cytoplasm and nuclei, and the appearance of the red colour in the appressorium, the remaining part of the germ-tube was not cut off by a septum (Figs. 4, *h*; 5, *k*). The formation of three or more small outgrowths or 'bulges' from the appressoria was often observed, and it was thought that these were substomatal vesicle initials (Figs. 4, *h*; 5, *h*). Usually two or three parallel nuclear divisions occurred in normal appressoria (Figs. 4, *g*; 5, *g*) and the four

TABLE

A Comparison between Appressoria and Substomatal Vesicles as formed on Artificial Membranes and as formed in the Stomata of Wheat Leaves. (The description and sizes given by Pole Evans (1907) are shown in the last column)

	On an artificial membrane				In stomata of Wheat leaves				Data of Pole Evans.			
	Form.	Size.	Colour	Number of infecting hyphae.	Form.	Size	Colour	Number of infecting hyphae	Form.	Size.	Number of infecting hyphae.	
<i>P. tritici.</i>	Appressorium	Oval	—	—	Cylindrical	—	Tinted orange-red	—	Well defined	—	—	
	Substomatal vesicle	Pear-shaped	$22 \times 15 \mu$	1	Pear-shaped	$20 \times 14 \mu$	Orange-red	1	Spherical	$22 \times 12 \times 15 \mu$	1	
<i>P. graminis.</i>	Appressorium	Oval	—	—	Cylindrical	—	Tinted brown-red	—	Well defined	$9 \times 27 \mu$	—	
	Substomatal vesicle	Cylindrical	$10 \times 30 \mu$	1 or 2	"	$10 \times 25 \mu$	"	1 or 2	Cylindrical	$9 \times 27 \mu$	1 or 2	
<i>P. glumarum.</i>	Appressorium	"	Yellow	—	"	—	Tinted yellow	—	Not clearly defined	—	—	
	Substomatal vesicle	Round	20μ	1	Round	17μ	Yellow	1	Oval	$8-19 \mu$	1	

or eight nuclei migrated with all the cytoplasm into the young substomatal vesicle. A septum was formed between the fully developed vesicle and the appressorium (Figs. 4, *f*, *g*; 5, *k*). In germ-tubes on membranes with waxes of lower congealing points, chromatin extrusions were numerous and substomatal vesicles did not develop. In appressoria on membranes with waxes of higher congealing-points, nuclear division was continued in the substomatal vesicle, and as many as twenty nuclei have been counted, aggregated towards its top. No infecting hyphae were formed by such substomatal vesicles, which were large, cylindrical, and often had no well-defined stalk. Separation from the appressorium by a septum did occur, but when this septum was absent a distinction between appressorium and substomatal vesicle became impossible, particularly in *P. graminis*. Few nuclear divisions took place in *P. graminis*, not more than four nuclei having been seen (Fig. 5, *k*). When the infecting hyphae grew out from the substomatal vesicle the migration in pairs of two, or of all, the nuclei occurred (Fig. 5, *k*). Frequently only one infection hypha developed in *P. graminis*, and then one pair of nuclei remained in the substomatal vesicle (Fig. 5, *k*). When 'drumsticks' were formed sometimes only two nuclei moved into them, but more usually all four made their way into the ball-like head (Fig. 4, *k*). In it the number of nuclei was reduced to one, though how this occurred was not certain (Fig. 4, *k*). Allen (1926) in her account of haustorial formation spoke of the degeneration of all but one nucleus. In these preparations the appearance suggested that nuclear fusion had taken place. This again suggested the possible homology of the drumsticks with haustoria. No example has been seen of more than one septum in the germ-tube, but septa were found at times in the infecting hyphae, and in each cell thus formed two nuclei were present (Fig. 4, *h*).

EXPERIMENTS ON LEAF INFECTION

Some experiments have been made to find out whether the infection hyphae of *P. tritici*, formed by substomatal vesicles on a membrane, would produce haustoria in the mesophyll cells of a leaf. Only the first-formed leaves of seedlings of *T. vulgare* were used, and all the experiments were carried out in a humidity chamber (R.H. *c.* 98 per cent.). First the lower epidermis was removed (for description of method see Part III), and the leaves were then cut into lengths of about $\frac{1}{2}$ in. and placed, mesophyll upwards, on drops of fresh 1.5 per cent. agar on microscope slides. After preparing a number of such leaf-strips, a selection was made of those whose exposed mesophyll showed no sign of being wet. The remainder were discarded.

Two types of 0.4 per cent. collodion membrane were employed. The first contained 0.05 per cent. of a wax of high congealing-point (52° C.), the second 0.05 per cent. of a wax of low congealing-point (36° C.). These membranes were made and mounted as previously described, and spores dropped on them. After 16 hours in a humidity chamber the membranes were examined under a microscope. Numerous substomatal vesicles were present on those membranes which contained the wax of high congealing-point and none on the

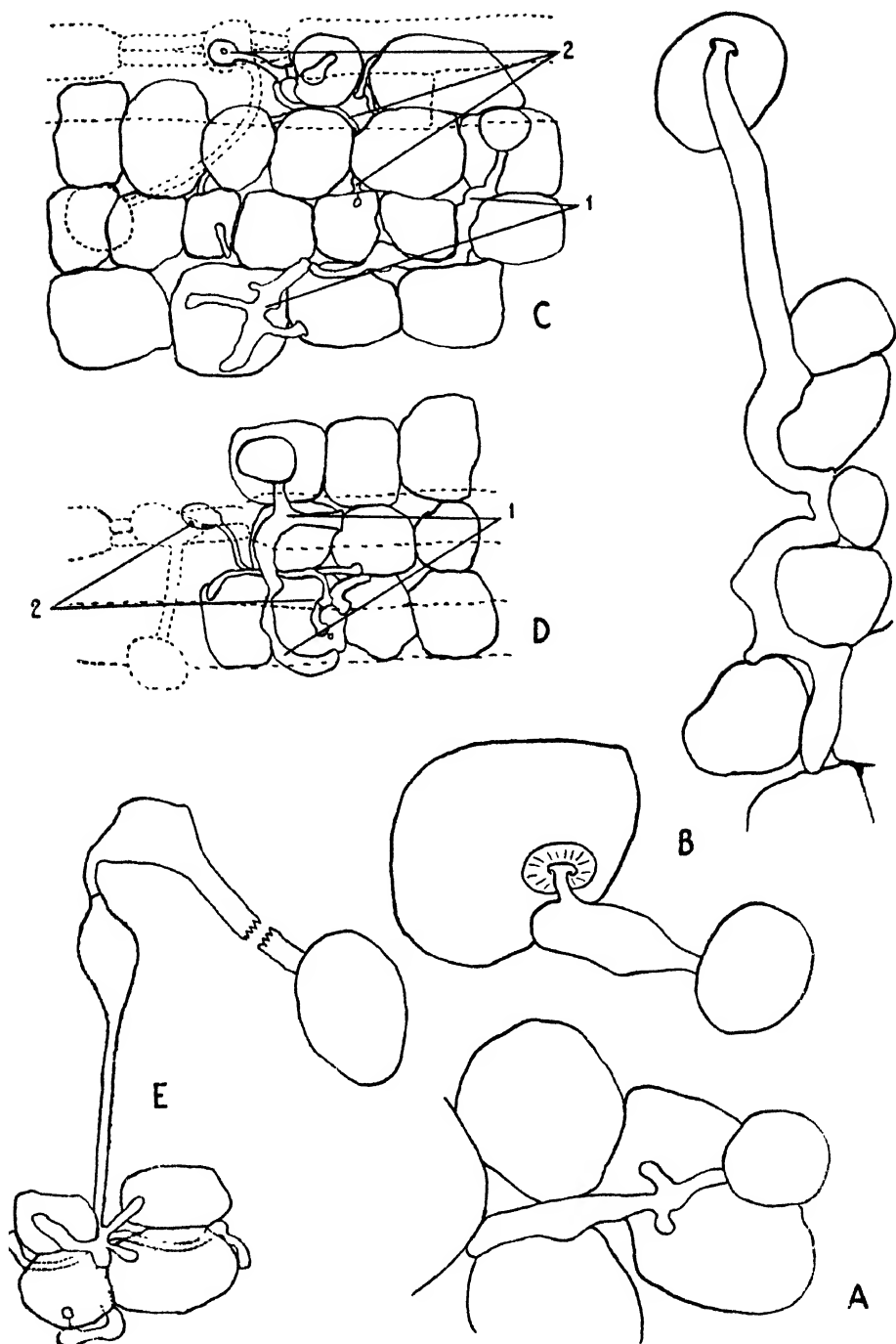


FIG. 6 (*See opposite*)

others. Each membrane was then carefully placed on a separate leaf-strip with the spores and their germ-tubes in contact with the exposed mesophyll cells. The part of the membrane not required was torn away. Great care was taken not to allow any part of a membrane or spores to touch any leaf tissue other than exposed mesophyll cells. After a further 48 hours in the humidity chamber the leaf-strips were stained in cotton blue dissolved in lactophenol and mounted in lactophenol and glycerine for microscopical examination.

On examining those leaf-strips in contact with a membrane containing a wax of low congealing-point, it was found that the germ-tubes in contact with the mesophyll cells had a large diameter and irregular outline (Fig. 6, *a*). They resembled the germ-tubes shown in Fig. 2, *b*, *c*, *d*. Their apices were somewhat swollen and contained many reddish-orange granules. No signs of septation, of haustorial mother-cells, or of penetration of the cell-walls of the mesophyll were observed. The apices of the germ-tubes and the adjacent mesophyll cell-walls were stained a deep blue. This was in sharp contrast to the light blue staining of the remaining mesophyll cells and of the germ-tubes not in contact with them.

On the leaf-strips in contact with a membrane containing a wax of high congealing-point, many appressoria and substomatal vesicles were observed. In a number of strips the infecting hyphae arising from the substomatal vesicles were seen to have formed haustoria in the mesophyll cells (Fig. 6, *e*). No haustoria were found in any of the other mesophyll cells. Septation and branching were common in these infecting hyphae. There were numerous reddish-orange granules in the substomatal vesicles and in those infecting hyphae not in contact with mesophyll cells. No such granules were seen in the infecting hyphae in contact with mesophyll cells. The infecting hyphae, substomatal vesicles, and mesophyll cells were all stained a light blue. In the few cases in which a germ-tube had not formed a substomatal vesicle, and had come into contact with a mesophyll cell, the same deep blue stain was seen as that in germ-tubes and the adjacent cells on membranes with a wax of low congealing-point.

A further experiment was made in which only part of the lower epidermis of the leaf was removed. As before, the leaves were then cut into convenient lengths and put with the mesophyll upwards on agar on microscope slides in a humidity chamber. The leaf-strips with wet mesophyll were discarded.

FIG. 6. Drawings of *P. tritici* germ-tubes. *a*, Germ-tubes growing amongst mesophyll cells. *b*, Germ-tube in close contact with a mesophyll cell. No penetration of the cell-wall had taken place. *c*, 1. A germ-tube coming directly in contact with mesophyll cells has failed to penetrate and form haustoria in them; 2. Successful 'infection', as shown by the presence of haustoria, has followed the entrance of a germ-tube through a stoma. Both germinating spores are lying on exposed mesophyll cells. *d*, 1. A germ-tube having come directly in contact with mesophyll cells below the uninjured epidermis has failed to form haustoria in them; 2. Successful 'infection', following entrance through a stoma, of a mesophyll cell, already the object of an unsuccessful attack. *e*, Successful 'infection' of exposed mesophyll cells by an infection hypha. The parent substomatal vesicle was formed on a membrane made from a 0.4 per cent. 9/91 collodion paraffin-wax mixture. In drawings *c* and *d* the uninjured epidermis, the spores lying on it, their germ-tubes and appressoria, are shown in dotted outline.

The edge of the exposed mesophyll was then brushed very gently with a camel-hair brush on which were spores of *P. triticina*. As a result some spores were deposited on the exposed mesophyll and others on the adjacent uninjured epidermis. The leaf-strips were left for 48 hours and afterwards stained in cotton blue dissolved in lactophenol and mounted as before for examination.

A high proportion of the spores germinated, both on the epidermis and on the exposed mesophyll. It was found that when germ-tubes entered the leaf through the stomata, normal appressoria, substomatal vesicles, and infection hyphae were formed. There were reddish-orange granules only in the substomatal vesicles, and the infection hyphae were septate and branched. In most cases the infection hyphae were traced from substomatal vesicles to the adjacent mesophyll cells, in which they had formed haustoria. Such haustoria were seen in mesophyll cells below the uninjured epidermis as well as in those cells from which the epidermis had been removed (Fig. 6, *c*, *d*). The germ-tubes from some spores came into direct contact with the mesophyll cells. In such cases no substomatal vesicles were formed. Instead their diameters were enlarged and their outline irregular. Their apices were swollen and contained reddish-orange granules. No septation or true branching or penetration of the mesophyll cell-walls was observed. Sometimes appressorial-like bodies were seen (Fig. 6, *b*). Those parts of the germ-tubes in contact with the mesophyll cells and the adjacent cell-walls were stained a deep blue, but all the remaining cells, germ-tubes, and infection hyphae were stained a light blue. Some examples were seen of germ-tubes and infecting hyphae in contact with the same mesophyll cell. In Fig. 6, *d*, is shown such a cell, which has a haustorium in it. The germ-tube (Fig. 6, *d*, 1), without passing through a stoma, came into contact with the cell and has not penetrated it. The parent germ-tube (Fig. 6, *d*, 2) of the infecting hyphae formed an appressorium and substomatal vesicle after contact with a stoma. The infecting hyphae grew and after contact with the cell penetrated it and formed a haustorium in it.

DISCUSSION

In the experiments described above the germ-tubes of *P. triticina*, *P. graminis*, and *P. glumarum* formed appressoria, substomatal vesicles, and infection hyphae after coming in contact with certain membranes. The differences between the species noted by Pole Evans (1901) and Allen (1926), as well as colour differences, were observed in the appropriate structures formed on the membranes. It has been shown that the infection hyphae, produced by *P. triticina* germ-tubes after contact with a membrane, will penetrate and form haustoria in the mesophyll cells of a wheat leaf.

The similarity between the stagshorn effect seen by Sappin Trouffy (1896) and others (Ezekiel 1920) and the incipient branching described here was patent and suggested that they were the same phenomenon. No mention was made by previous workers as to whether this stagshorn effect was correlated with any of the conditions of germination, though Ezekiel (1920) found it to

occur more frequently in some strains of *P. graminis*. It would seem possible that the agar or water surface, on which germination had occurred, had provided the required stimulus.¹

The behaviour of these germ-tubes would suggest that there was an optimum for stimulus and response, the position of this optimum being indicated by substomatal vesicle formation. Below the optimum, branching, with initiation only of nuclear division, took place. The initiation only of nuclear division below the optimum, and completion at the optimum, would suggest an increasing intensity of stimulus until the optimum was reached. Above the optimum the appressoria were small and did not form substomatal vesicles. The continual formation of new growing-points might be due to lack of nuclear division and septa formation. It would seem probable that the stimulus which induced the formation of growing-points was different from that which induced appressorial formation.

The swelling and bursting of the germ-tubes were frequently seen on some membranes whether penetration had occurred or not. They were seldom found when the stimulus for nuclear division was near the optimum or was well away from the optimum. Therefore it seemed that bursting was to be associated with unsuccessful nuclear division rather than with failure to penetrate the underlying membrane. On the other hand, the observation that more or less perfect nuclei were present in the cytoplasmic debris after a burst would make such a suggestion unlikely.

Allen's description (1926) of the formation of haustorial mother-cells and of haustoria by rust hyphae in the leaf would suggest that they are formed as a reaction to a contact stimulus. Were this suggestion correct, then the formation of both haustoria and substomatal vesicles would be due to contact stimuli. There was between them a morphological similarity emphasized on thin membranes when penetration usually took place with the substomatal vesicle lying in the liquid below the membrane. The appressoria corresponded to the haustorial mother-cells, and the substomatal vesicles to the haustoria, while the membrane occupied the same position as the mesophyll cell-wall. The main difference, other than that of size, has been the lack of septa formation in the germ-tubes. All the protoplasm of the germ-tubes, probably owing to this absence of septa, passed into the substomatal vesicles, and no further growth took place above the membrane.

The difference in behaviour of the infection hyphae and the germ-tubes on coming into contact with the mesophyll cells of a leaf has been described. Other differences, such as nuclear division and septation in the infection hyphae and not in the germ tubes, &c., have been mentioned. As these differences were seen after contact with the same mesophyll cell or membrane, they would appear to be differences in reaction to the same contact stimulus. The change from germ-tube to infection hypha takes place when the substomatal vesicle is formed.

¹ Since this paper was written, Hurd-Karrer and Rodenhiser (Amer. J. Bot., 1947, xxxiv. 377-84) have described substomatal vesicle formation on certain agar media.

SUMMARY

The phenomena, exhibited by the germinating uredospores of three rust fungi as they enter the living host plant, have been observed in germ-tubes growing on specially prepared, artificial membranes. These phenomena—namely formation of appressoria, of substomatal vesicles, and of infection hyphae—are shown to be induced by a contact stimulus. Certain other phenomena, in particular nuclear division, seem also to be the result of a contact stimulus. Infection hyphae of one rust formed on such membranes are shown to produce haustoria in living mesophyll cells of a wheat leaf.

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ERRATUM

DICKINSON: *Studies in the Physiology of Obligate Parasitism. Part I.*

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Note

The absorption of nitrogen by the sugar-cane plant at different stages of growth.—The rate of uptake of nutrients by plants appears to be related to the stage of its growth and development, and physiological analysis of this relationship has a scientific as well as a practical interest. Observations (Nelson, 1946) on several crop plants show that nearly 75 per cent. of the total amount of nutrients taken up by the plant is absorbed before flowering. In some plants like tomato (Hester, 1938), soy-bean (Austin, 1935), and peas (Maximov, 1938) such a relationship is not indicated, and Maximov (1938) attributes this divergent behaviour to the more or less continuous type of development in these plants.

Gregory (1937) has outlined an analysis of this relationship from his extensive observations on the growth and nutrition of barley. According to him the requirements of nutrients for growth and maintenance tend to an exponential increase with a similar increase in the number of primordia, and the critical phase at which demand overtakes supply marks the maximum in rate of uptake. This point marks the beginning of internal starvation. Even with a continuous nutrient supply internal starvation supervenes nevertheless. Internal starvation in barley is due, according to Gregory, to a sudden fall in the rate of uptake by the roots, and this stage is also marked by the onset of the reproductive phase.

The difference between the growth habit of sugar-cane and a cereal like barley may be briefly noted. In barley first only meristematic growth takes place in the stem and only after all its internodes, as well as its inflorescence, have been laid down does elongation take place. In sugar-cane, on the other hand, after an initial period during which the increase in height is mainly due to elongation of leaf sheaths, stem internodes start elongating and then follows a sort of continuous (up to a limit) differentiation and development of leaves and stem internodes. Flower initials do not appear to have been laid down at the time stem internodes commence elongating. In view of this difference it appeared interesting to investigate the trend of absorption of nutrients by sugar-cane.

The writer, during study of the growth of sugar-cane under field conditions at Pusa, north Bihar, made observations on the uptake of nitrogen at various stages of its life-cycle. The observations, which relate to the variety Co 313, were made during four seasons and in six separate field experiments. It may be noted that the sugar-cane crop occupies the land in north Bihar for about a year from the time of planting to harvest. The data under consideration relate to unmanured control plots. It is not proposed to discuss here final yields or manurial effects. The first sample of plants was collected about 8 weeks after planting (when germination was complete), and subsequent samples were collected at intervals of 4 weeks until shoot elongation practically ceased. Each sample consisted of twelve clumps. Since the dates of planting were not identical in all the seasons, the periodical observations were adjusted to the same time-scale by a graphical method. Smooth curves were prepared for each season separately and the values at the age of 10, 12, 14 weeks, &c., were read off from the graphs. Mean curves were then prepared from these values (Fig. 1).

From Fig. 1 it is seen that the leaf-weight curve is of the ubiquitous sans serif shape. After about 28 weeks there was no substantial increase in leaf weight, and it

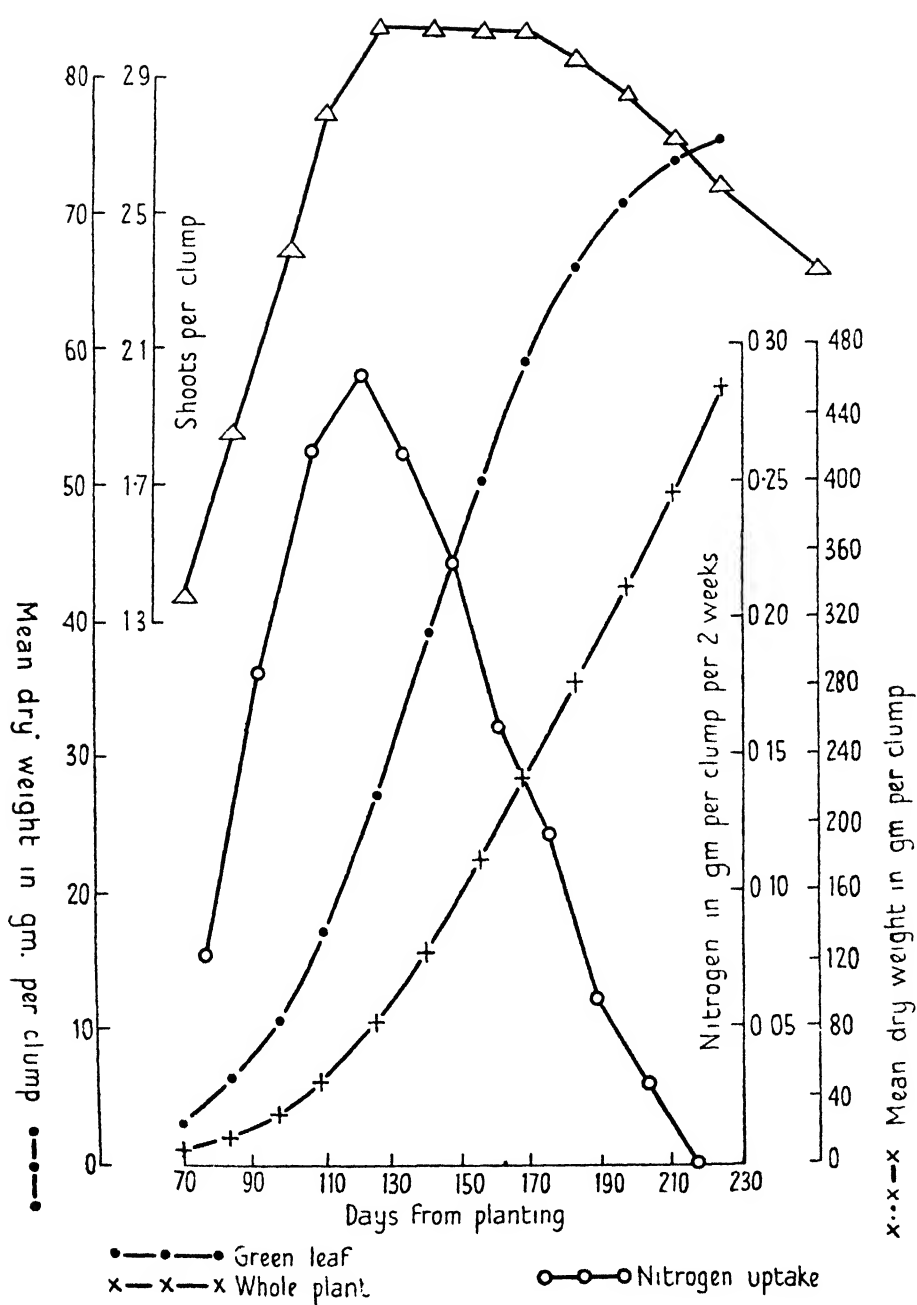


FIG. 1.

may be concluded that leaf growth ceased about 4 weeks earlier than the growth of the plant as a whole. Tillering continued to increase up to 18 weeks, by which time the maximum was reached; a more or less steady level was then maintained for another 6 weeks, and subsequently there was a steady decline as the rate of mortality exceeded the rate of production. Maximum leaf weight was attained about 4 weeks after the peak level of tillering declined.

The absolute rate of uptake of nitrogen (Fig. 1) increased to a maximum in about 17 weeks and thereafter declined steadily and reached the zero level after about 28 weeks. Maximum leaf weight was also attained at about 28 weeks; thus after 28 weeks there was little uptake of nitrogen and little leaf growth. It may also be noted that the maximum point on the nitrogen uptake curve corresponds in time with the inflexion on the leaf-weight curve.

It may not be out of place to refer to the observations of Das (1936) on the uptake of nitrogen by sugar-cane in Hawaii, where it is grown as a biennial crop. The writer calculated the rates of uptake from the curves in Fig. 22 of this paper and found that the highest rate of uptake occurred between $3\frac{1}{2}$ and 6 months after planting, at low and medium levels of nitrogen. At the high level of nitrogen the rate of uptake was higher at a later stage between $14\frac{1}{2}$ and $16\frac{1}{2}$ months than the earlier one between $3\frac{1}{2}$ and 6 months, but there appears to be some discrepancy in this case as no nitrogen was taken up during the interval between $12\frac{1}{2}$ and $14\frac{1}{2}$ months. It may be noted that in this case also cane formation commenced between $3\frac{1}{2}$ and 6 months.

According to Gregory (1937) the sudden fall in the rate of uptake is related to the onset of the reproductive phase. Crowther (1934) has suggested that with the onset of flowering and fruiting in cotton carbohydrates are diverted more towards the developing bolls, the root growth is thus inhibited due to carbohydrate shortage, and with it the uptake of nitrogen diminishes. Eaton and Joham (1944) obtained a practical verification of this theory by their defruiting experiments on cotton. It is not clear, however, how the diversion of the supply of carbohydrates away from the root can account for the sudden fall in the rate of uptake of nutrients in the case of barley. The flowering spike is in a very rudimentary stage at this time, and the requirement of carbohydrates for its growth cannot conceivably be large. Archbold and Mukherjee (1942) have obtained some data on the carbohydrate content of the root of barley at various stages of growth in an attempt to assess the contributions of different plant parts to the development and ripening of the ear. These data show, however, a general rise in concentration of total sugar in the root until ear emergence.

Flowering in sugar-cane, under the conditions of these experiments, was, however, very irregular and erratic. Again, at the age of 17 weeks, no flower primordia were discovered in the clumps that were sampled. The crop was usually 'earthed up' about this time after the first monsoon shower, and as the surface roots were destroyed the rate of uptake was probably diminished thereby. Soon after, however, new roots developed from the nodes that got buried and the rate of uptake might not, therefore, be expected to show a consistently declining trend apart from a temporary break. Again, if this were indeed the sole reason for the sharp break in the curve of uptake the unearthed crop might show considerable superiority due to non-interference with the root system. Experience does not show this to be the case, and one may, therefore, reasonably infer that this trend in the uptake of nitrogen is a natural consequence of the physiological processes of the plant.

Approximately 14 weeks after planting the stem internodes commenced elongating as well as storing sugar. It was observed that the correlation coefficient between stem height and plant height reached the significant value of $+0.956$ at about 14 weeks from planting. By 17 weeks the stem contained about 60 per cent. of the total sugar content of the plant (Table). These data were collected from mother

TABLE

Days from planting.	Sampling date.	Plant height (in.).	¹ Plant and stem height.	Distribution of total sugar percentage as		
				Leaf.	Sheath.	Stem.
54	24/4	16.3	—	—	—	—
75	15/5	27.1	$+0.104$	52.6	37.5	9.9
96	5/6	40.9	$+0.956$	—	—	—
117	26/6	80.9	$+0.973$	23.7	14.1	62.2
159	7/8	—	$+0.984$	8.9	9.4	81.7

¹ Correlation coefficient.

stalks only. The dry weight of stem at 117 days formed only 25 per cent. of the total weight of the plant, whereas 60 per cent. of the total sugar content of the plant was located in it. Due to the obvious difficulty of collecting root samples in the field, data on the sugar content are not available, and it would seem pointless to speculate from the partition of sugars in the aerial parts whether the root suffered from deficiency of carbohydrate. In any case these observations, as well as those on barley (Gregory, 1937) and potato (Hawkins, 1946), point to some sort of a regulating mechanism, presumably hormonal, and suggest another mode of approach to the problem of solute absorption by the root to which considerable attention, from the metabolic point of view, has been given in recent years by Hoagland, Lundegårdh, Steward, and others.

These observations were carried out at the Central Sugar-cane Research Station, Bihar, Pusa. The writer's thanks are due to K. L. Khanna, Sugar-cane Specialist, for encouragement and interest and to Mr. Mustafa Karim, M.Sc., Junior Assisant, for nitrogen analysis and general assistance.

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Growth and Heterocyst Production in *Anabaena cylindrica* Lemm.

II. In Relation to Carbon and Nitrogen Metabolism

BY

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With eight Figures in the Text

DURING the growth in culture of *Anabaena cylindrica* heterocyst frequency varies in a characteristic manner and is inversely related to the concentration of combined nitrogen in the organism (Fogg, 1944). Following a line of inquiry suggested by this finding, an investigation has been made of the effect on heterocyst production by this alga of conditions likely to influence the balance between its carbon and nitrogen metabolism.

There appear to have been no previous studies of this kind. De (1938), incidentally during his work on nitrogen fixation, recorded heterocyst abundance in species of *Anabaena* grown under various nutritional conditions, but his observations were made on senescent cultures and do not throw much light on the nature of the conditions leading to the formation of these structures.

METHODS

Experiments were carried out by comparing the course of heterocyst production in cultures supplied with the substances or subjected to the conditions, the effects of which were to be investigated, with that in control cultures. Since variation in heterocyst numbers is most marked in the exponential phase of growth, observations were made chiefly on young cultures. The primary data were recorded as *heterocyst frequencies*, i.e. number of heterocysts expressed as a percentage of the total number of cells counted. Heterocysts may vary in dimensions as well as in frequency, but their individual growth may be regarded as a separate phenomenon from their formation, and such variations have not been considered in this study. Canabaeus (1929) has investigated the effect of some external conditions on heterocyst size.

Changes in heterocyst frequency depend on the relative increases in numbers of heterocysts and of normal cells. Consequently, in cultures initially similar, comparisons of heterocyst frequencies after equal periods of time are not valid if the treatments applied have affected the rate of growth in cell numbers to differing extents. In interpreting results it has, therefore, been found most useful to employ the method of Huxley (1932) for studying differential growth ratios. Over any short period of time the total number of

heterocysts per unit volume of culture medium (h) may be regarded as related to the total number of normal cells in the same volume (n)¹ according to the expression

$$\log_e h = a + k \log_e n,$$

where a and k are constants. k is a measure of the production of heterocysts that is independent of variations in general growth rate and it may be used in the comparison of cultures subjected to different treatments. During the exponential growth phase of *Anabaena*, k does not remain constant but varies in a characteristic manner, the curve obtained on double logarithmic plotting of h against n , the slope of which is equal to k , being obliquely sigmoid (see, for instance, Fig. 4).

Details of technique. The strain of *Anabaena cylindrica* Lemm. and, in general, the methods used, were the same as in previous work (Fogg, 1942, 1944). The following modifications of technique should, however, be noted.

Culture methods. An improved medium of the following composition, with which soil extract is not needed for the maintenance of stock cultures, was employed: K_2HPO_4 , 0.2 g.; $MgSO_4 \cdot 7H_2O$, 0.2 g.; $CaCl_2$, 0.1 g.; Fe, as chloride, 0.4 mg.; Mn, as chloride, 0.1 mg.; Mo, as sodium salt, 0.1 mg.; B, as boric acid, 0.1 mg.; Cu, as sulphate, 0.01 mg.; Zn, as sulphate, 0.01 mg.; 'Pyrex' distilled water, 1.0 litre. When other substances were added to this basal medium the reaction was adjusted to its original value, i.e. pH 7.4.

Bortels (1940) has shown that molybdenum is necessary for the growth of *Anabaena cylindrica* in the absence of available combined nitrogen. Using the medium without the additional trace elements it was not possible to confirm this finding (Fogg, 1942), but with the improved medium omission of the element leads to reduced growth. In an experiment to demonstrate this, growth of *Anabaena*, inoculated from a culture to which no molybdenum had been supplied, was compared in media containing 0.1 p.p.m. of molybdenum and without added molybdenum. Tests on the concentrated basal medium with $\alpha\alpha'$ -dipyridyl by the method of Komarovski and Poluektov (Johnson and collaborators, 1943) showed that the molybdenum present as impurity in the second of these media could not be greater in amount than 0.005 p.p.m. After 30 days growth was determined as total nitrogen by micro-Kjeldahl analysis. The results, each the mean of three determinations, were 0.438 ± 0.0035 mg. and 0.257 ± 0.010 mg. respectively for the cultures with and without added molybdenum. Growth in the latter series was yellowish, whereas that in the former was healthy.

Since a culture chamber was not available in the earlier stages of this investigation, in certain experiments the culture flasks were incubated at room temperature arranged at equal distances around 100-watt pearl lamps. Later, an apparatus described elsewhere (Fogg, 1948), in which cultures could be grown under conditions of constant temperature and uniform illumination, became available. Since they were thus not all obtained under the same

¹ Since heterocysts form only a small fraction of the total number of cells of both kinds, it is sufficiently accurate to take $n \sim n + h$.

standard conditions and are therefore not directly comparable, the main results obtained have been confirmed in repeat experiments. Light intensities were determined in the plane of the surface of the medium with an Everett Edgcumbe 'Autophotometer'.

Estimation of growth rate. Growth was followed by measurement of filament length. Bristol Roach-type culture vessels (Fogg, 1944) were not employed; instead, cultures were grown in 'Pyrex' conical flasks plugged with cotton-wool and determinations made, in the early stages of growth up to the fourth day, on samples withdrawn aseptically after shaking and, later, on whole cultures. Results obtained by this method are not so accurate as those obtained by the technique previously used, but the simplification in manipulation enables more cultures to be used in a single experiment. Determinations of filament length per unit volume of culture medium were made with a 16-mm. objective and $\times 10$ eye-piece in conjunction with a Fuchs-Rosenthal counting-cell ruled in $\frac{1}{16}$ -mm. squares and 0.2 mm. deep. Where cell numbers were required, they were calculated from values for filament length obtained by interpolation from growth curves and estimations of mean cell length.

Estimation of heterocyst frequency. A 4-mm. objective and $\times 10$ eyepiece were used. Since at certain times all stages between normal cells and heterocysts can be observed, it is necessary to have some arbitrary distinction between the two. In this work a cell has not been counted as a heterocyst unless it showed a well-defined thickened wall.

Statistical methods. These are the same as those used in previous work (Fogg, 1944). In the results given below each value for heterocyst frequency is the mean of determinations made on two separate cultures. Significant differences at the $P = 0.05$ level between two such means (d'), calculated from the mean square for error for the whole of the data and the appropriate value of t , are indicated on the graph for each experiment. The symbol, d' , is also used elsewhere to denote a significant difference at the $P = 0.05$ level.

EXPERIMENTAL RESULTS

The effect of ammonium salts. Ammonium nitrogen is readily available to *Anabaena*. Numerous experiments have shown that its presence in the culture medium markedly inhibits the formation of heterocysts. Neither Glade (1914) nor Maertens (1914), both of whom studied the effect of ammonium salts on the growth of blue-green algae, remark on this effect.

Some representative results are given in Figs. 1 and 2. Experimental details were as follows: media, (1) basal, (2) basal + 0.0005 M ammonium chloride, distributed in 25-ml. portions in 100-ml. flasks; inoculum, from a 20-day stock culture; temp., 27° C.; light intensity, 1,600 metre-candles. Growth in filament length (curve *A*) was not appreciably altered by the presence of ammonium chloride. During the period 0-6 days the relative growth constants (Fogg, 1944), each based on 9 determinations, were $R_c = 1.075 \pm 0.049$ and $R_a = 1.149 \pm 0.083$, for the control and ammonium cultures respectively.

The difference between these two values is not statistically significant, d' amounting to 19.2 per cent. of R_c . Ammonia determinations (curve B) were made directly on the filtered medium by a modification of Nessler's method (Folin and Denis, 1916). The small amount of ammonia apparently remaining

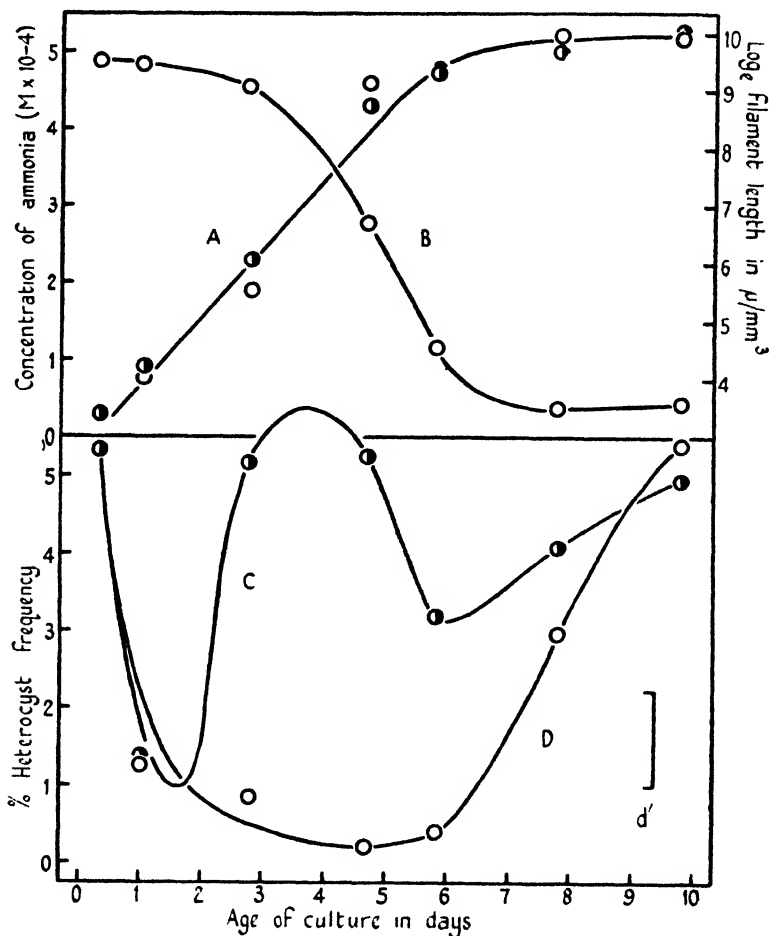


FIG. 1. Effect of ammonium chloride on growth and heterocyst production in *Anabaena cylindrica*. A, growth as \log_e filament length in μ/mm^3 of culture medium; B, concentration of ammonia in culture medium ($M \times 10^{-4}$); C, D, heterocyst frequency; \bullet , control series, \circ , ammonium series. Each point the mean of two determinations; d' , significant difference at $P = 0.05$ level between two values of heterocyst frequency.

in the older cultures was probably produced by decomposition during Nesslerization of nitrogenous substances excreted by the alga.

Whereas heterocyst frequency showed the usual variation in the control series (curve C), rising to a maximum at about the fourth day, in the series with ammonium chloride (curve D) it fell to a low value and remained there until about the seventh day, when ammonia had nearly disappeared from

the medium, after which it rose abruptly. All of the more important features of the two curves are statistically significant.

These data are analysed in Fig. 2 by double logarithmic plotting. The curve for the control series (*E*) is normal. For some while after inoculation $k \sim 0$, heterocyst numbers remaining nearly constant and their frequency falling. Following this was a phase in which $k \sim 3$ and both heterocyst

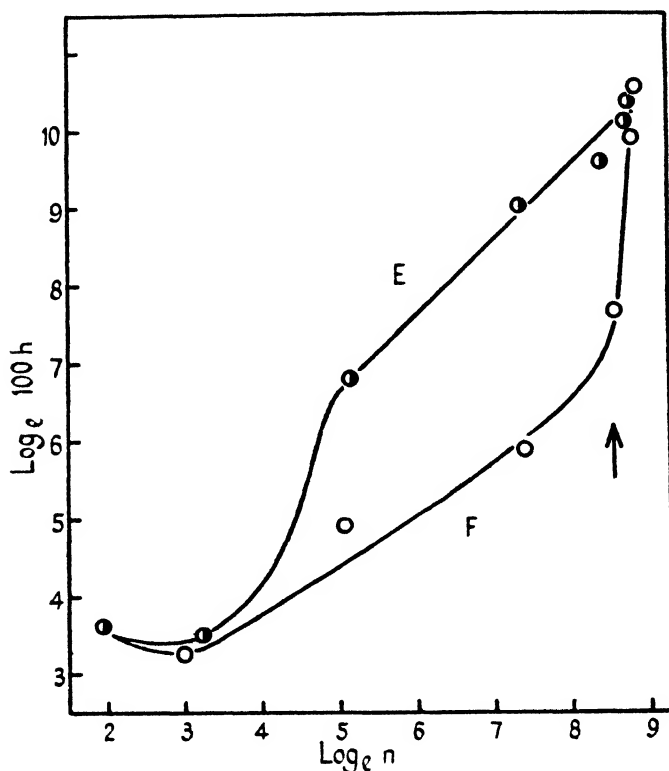


FIG. 2. Effect of ammonium chloride on heterocyst production in *Anabaena cylindrica*. $\text{Log}_e 100h$ plotted against $\text{log}_e n$. E, \bullet , control series; F, \circ , ammonium series. The arrow indicates the point at which ammonia was exhausted from the medium.

numbers and frequency rose abruptly. Finally k became about equal to unity and the curve approximates to a straight line. During this phase heterocyst frequency remained at a high value, its fluctuations reflecting minor variations in k . In the cultures with ammonium chloride (curve *F*), k remained at a more or less constant value, ~ 0.6 , after the initial period in which it approximated to zero. Heterocyst formation was not completely inhibited, since k had a finite value, but because it was less than unity heterocyst frequency progressively fell. At higher values of $\text{log}_e n$, k increased suddenly to $c. 11.0$ and the curve rises steeply until it joins that for the control series. The point of inflexion corresponds to the ammonia concentration falling below 0.00004 M. It is to be noted that when ammonia has virtually disappeared from the

medium the value for k does not become the same as in the control series at the corresponding stage, but much higher.

In cultures supplied with sufficient ammonium chloride inhibition of heterocyst formation, so that the frequency remained below about 0.25 per cent., has been observed to last up to 26 days. Such cultures became moribund since the pH of the medium fell from 7.4 to about 5.0. As soon as the ammonia was exhausted heterocyst formation increased abruptly, even if the medium was acid. Inhibition of heterocyst formation was also found to occur when diammonium hydrogen phosphate was used instead of the chloride.

Inhibition of heterocyst formation by ammonium nitrogen is perhaps a general phenomenon in the Myxophyceae since *Gloeotrichia natans* Rabenh. grown in impure culture in the presence of ammonium chloride showed complete absence of heterocysts, although these structures were present to the normal extent in otherwise similar cultures without added combined nitrogen.

Methylamine, added to the medium as hydrochloride, did not affect heterocyst formation in the same way as ammonium chloride. In cultures containing methylamine at an initial concentration of 0.001 M, growth was reduced and unhealthy, but, after falling to 0.11 per cent. on the seventh day, heterocyst frequency rose steadily to 1.5 per cent. on the seventeenth day, before the alga became completely moribund. The final pH of the medium was 6.8, indicating that little of the methylamine had been absorbed.

The effects of nitrate and of glucose. Nitrate forms a readily available source of nitrogen for *Anabaena* (Fogg, 1942). Glucose is evidently assimilated by the alga since growth in older cultures is increased in its presence.

In obtaining the results which are presented in Figs. 3 and 4, experimental details were as follows: media, (1) basal, (2) basal+0.028 M glucose, (3) basal+0.002 M potassium nitrate, distributed in 100-ml. portions in 250-ml. flasks; inoculum from 39-day culture; temp., 22–27° C.; light intensity, 900 metre-candles. Values for R , each based on 8 determinations of filament length, were found to be

Control	R_c ..	0.903 ± 0.045
Nitrate	R_n ..	1.005 ± 0.032
Glucose	R_g ..	0.884 ± 0.105

The difference between R_c and R_n is scarcely statistically significant ($t_{[12]} = 1.85$, $P < 0.1 > 0.05$; $d' = 13.3$ per cent. of R_c). R_g and R_c are not significantly different ($t_{[12]} = 0.166$, $P < 0.9 > 0.8$; $d' = 27.6$ per cent. of R_c). Other experiments, conducted under approximately similar conditions, have likewise failed to demonstrate any statistically significant effect of glucose or nitrate on the relative growth rate of *Anabaena* during the exponential phase.

During the last 3 days of the experiment heterocyst frequency was depressed in the presence of nitrate (curve C) and increased in the presence of glucose (curve D). The effect of nitrate is statistically significant. That of glucose is barely significant, but other experiments have shown the effect to be reproducible. In Fig. 4 the results are plotted on the double logarithmic

grid. All three curves are of the normal form. Two of the earlier points for the nitrate series (*F*) deviate somewhat from the fitted curve. At low heterocyst frequencies the error is proportionately much greater than at higher values, and there is no reason to suppose that the value of *k* departed very far

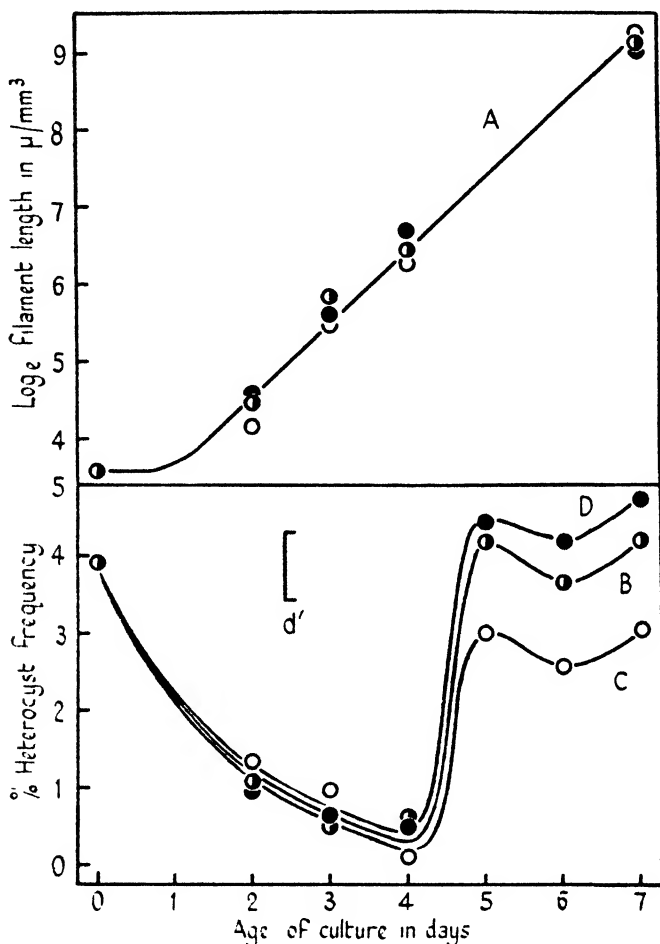


FIG. 3. Effects of nitrate and of glucose on growth and heterocyst production in *Anabaena cylindrica*. A, growth as \log_e filament length in μ/mm^3 of culture medium; B, C, D, heterocyst frequencies, \odot , control series, \circ , potassium nitrate series, \bullet , glucose series. Each point the mean of two determinations, *d'*, significant difference at $P = 0.05$ level between two values of heterocyst frequency.

from zero during this period. At higher values for $\log n$ the curve for the nitrate series (*F*) is displaced downwards and that for the glucose series (*G*) upwards with respect to that for the control series (*E*), *k* having about the same value in all three. It, therefore, seems that these substances do not affect heterocyst production in the later stages of the exponential phase and that their effect must be exerted during the first 5 days of growth. The results

available do not provide sufficient basis for deciding what variations in k are responsible.

The results of other experiments indicate that during the period of this experiment the change in nitrate concentration in the cultures supplied with this substance was of the order of 8 per cent. of the initial concentration.

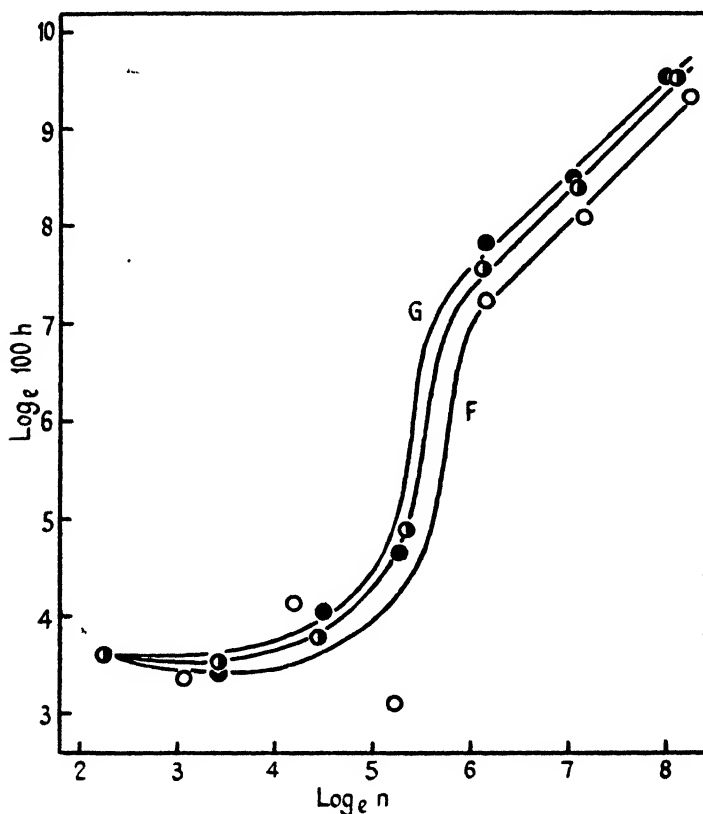


FIG. 4. Effects of nitrate and of glucose on heterocyst production in *Anabaena cylindrica*. $\text{Log}_e 100h$ plotted against $\text{log}_e n$; E, \bullet , control series; F, \circ , potassium nitrate series, G, \bullet , glucose series.

0.002 M potassium chloride added to the culture medium produces higher heterocyst frequencies at the end of the exponential phase (Canabaeus, 1929, has also noted that chlorides produce increases in heterocyst numbers). Hence it appears that the effect of potassium nitrate is due to the anion.

The effects of succinate, aspartate, and asparaginate. It seems probable that the compounds, succinic acid, aspartic acid and asparagine, play important parts in the metabolism of blue-green algae, as they are known to do in that of other organisms, and since they present a series having the same carbon skeleton but increasing amounts of nitrogen their effect on heterocyst production seemed worth investigating.

Some representative results are presented in Figs. 5 and 6; experimental details were as follows: media, (1) basal, (2) basal+0.00025 M succinate, (3) basal+0.00025 M asparaginate, distributed in 100-ml. portions in 250-ml.

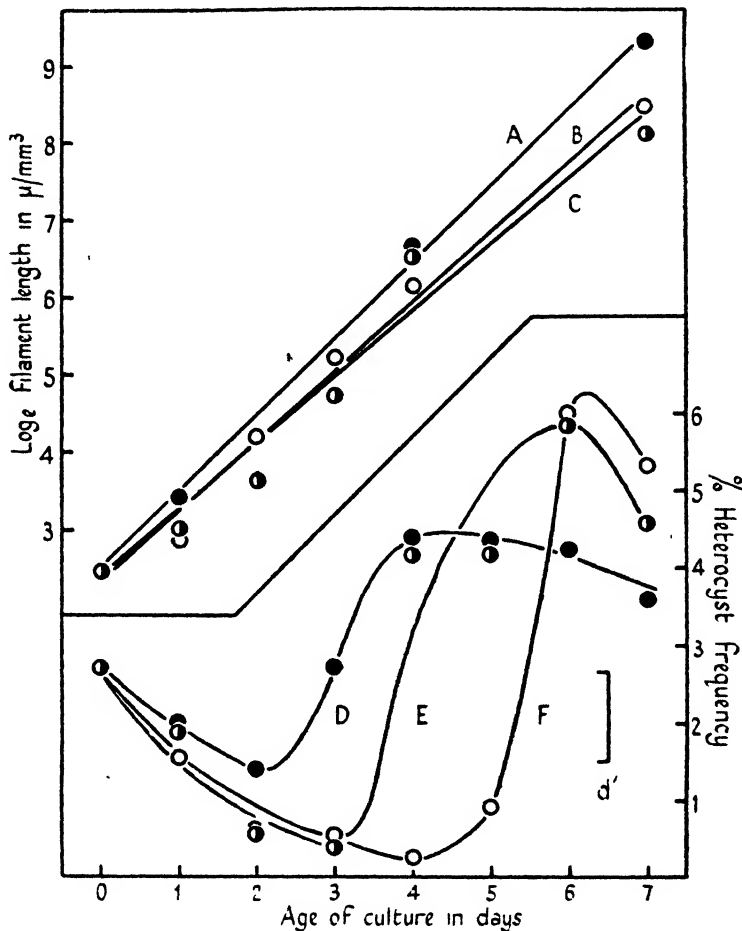


FIG. 5. Effects of succinate and of asparaginate on growth and heterocyst production in *Anabaena cylindrica*. A, B, C, growth as \log_e filament length in μ/mm^3 of culture medium; D, E, F, heterocyst frequency; ●, control series, ○, asparaginate series, ●, succinate series; each point the mean of two determinations; d' , significant difference at $P = 0.05$ level between two values of heterocyst frequency.

flasks; inoculum from a 9-day culture; temp., 15.5–25° C.; light intensity, 800 metre-candles. Relative growth rates, each based on 11 determinations of filament length (7 in the case of succinate), were found to be

Control	R_c ..	0.878 ± 0.102
Succinate	R_s ..	0.988 ± 0.059
Asparaginate	R_a ..	0.905 ± 0.074

There is no statistically significant difference between the above values for R .

Comparing R_s and R_c , $t_{[14]} = 0.932$, $P > 0.3 < 0.4$, $d' = 28.9$ per cent. of R_c . Comparing R_a and R_c , $t_{[18]} = 0.214$, $P > 0.8 < 0.9$. However, in another experiment at a higher light intensity, 1,700 metre-candles at 25°C ., the presence of asparaginate in the medium at the same concentration produced a statistically significant increase in relative growth rate. In this case

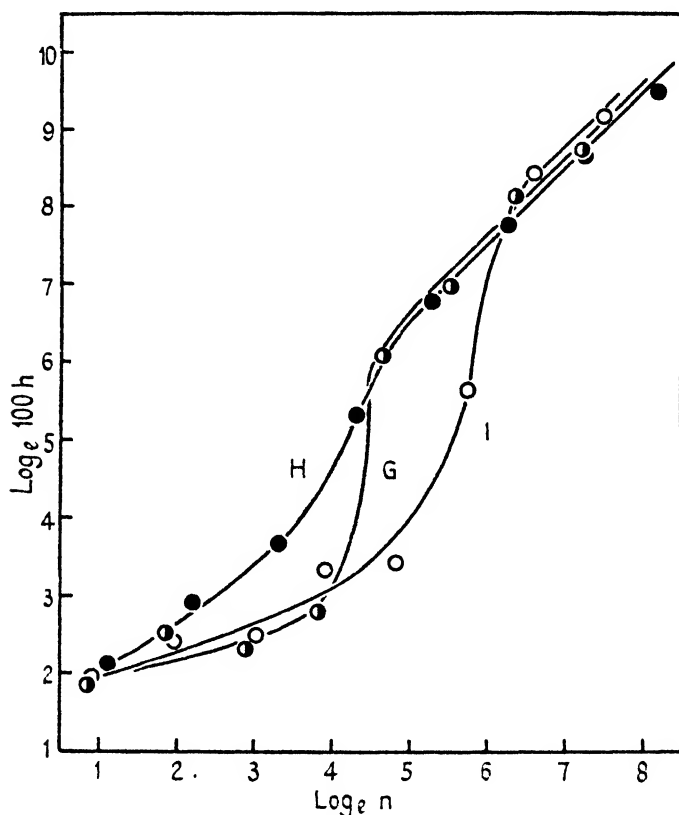


FIG. 6. Effects of succinate and of asparaginate on heterocyst production in *Anabaena cylindrica*. $\log_e 100h$ plotted against $\log_e n$, G, ●, control series; H, ●, succinate series; I, ○, asparaginate series.

$R_c = 1.070 \pm 0.106$, $R_a = 1.429 \pm 0.030$, $t_{[4]} = 3.26$, $P < 0.05 > 0.02$. At low light intensities asparaginate has a definitely inhibitory effect on growth. In the same experiment in a light intensity of 215 metre-candles, 0.00025 M asparaginate produced a progressive decrease in relative growth rate until after 9 days the density of alga, initially $6.4 \mu/\text{mm}^3$, was only $76 \mu/\text{mm}^3$ compared with $546 \mu/\text{mm}^3$ in control cultures. A possible explanation for this phenomenon is that at low light intensities the carbon residue of asparagine is utilized preferentially so that ammonia accumulates in toxic quantities within the cells.

0.00025 M aspartate has been found to inhibit growth of *Anabaena* completely. Urea at a concentration of 0.0005 M had a similar effect. Possibly the phenomenon is similar to that described by Audus and Quastel (1947),

who found that a number of amino-acids and amides, among them aspartic acid and urea, supplied in concentrations of this order, have an inhibitory effect on the growth of roots of cress seedlings.

Returning to the consideration of the results plotted in Fig. 5, it will be seen that with respect to that for the control series (*E*) the curve of heterocyst frequency for the succinate series (*D*) rises earlier but reaches a lower level in the older cultures, whereas in that for asparaginate (*F*) the rise is postponed but a higher level is eventually reached. The more important differences between the curves are statistically significant with the exception of the final rise of the heterocyst frequency in the asparaginate series above the value for the control series. However, this feature has been reproduced in two other experiments, as have the other differences. The double logarithmic plot (Fig. 6) shows that the sequence of changes in k was normal in all three series, the differences between them lying in the stage at which the abrupt rise in k took place. In the succinate series (curve *H*) this rise took place early so that the curve does not depart very far from a straight line. In the case of asparaginate (curve *I*) the rise is postponed but was greater when it did occur. At higher values of $\log n$, k is unaffected by the presence of succinate or asparaginate.

The rise in the value of k found in the asparaginate series is not caused by the exhaustion of that substance from the medium. In a separate experiment the disappearance of asparagine in *Anabaena* cultures was followed. Amide nitrogen remaining in the medium was determined by titration as ammonia after hydrolysis of the concentrated medium with N sulphuric acid for 2 hours and distillation with magnesium oxide. From the results obtained it seems that about 58 per cent. of the initial amount of asparagine must still have been present at the end of the experiment described above. No ammonium nitrogen could be detected in asparaginate cultures.

Germinating heterocysts have been observed occasionally during the first few days of growth in cultures supplied with succinate. This phenomenon has not been noticed under any of the other experimental conditions used in the course of this work.

The effect of glycine. The effect of glycine on growth and heterocyst production was investigated in an experiment carried out at 25° C. in light intensities of 1,600 and 430 metre-candles. In both series its effect was similar to that of asparagine described in the previous section.

The effect of light intensity. Exposure to different light intensities may be expected to produce differences in the balance between carbon and nitrogen metabolism.

Figs. 7 and 8 are based on results obtained in an experiment in which the details were as follows: basal medium, without addition, distributed in 100-ml. portions in 250-ml. flasks; inoculum from 34-day culture; temp., 25° C.; light intensities, 260, 1,300, and 3,100 metre-candles. In this case it was necessary to measure the light intensity in a plane normal to the incident light and at right angles to the surface of the medium. As will be seen from

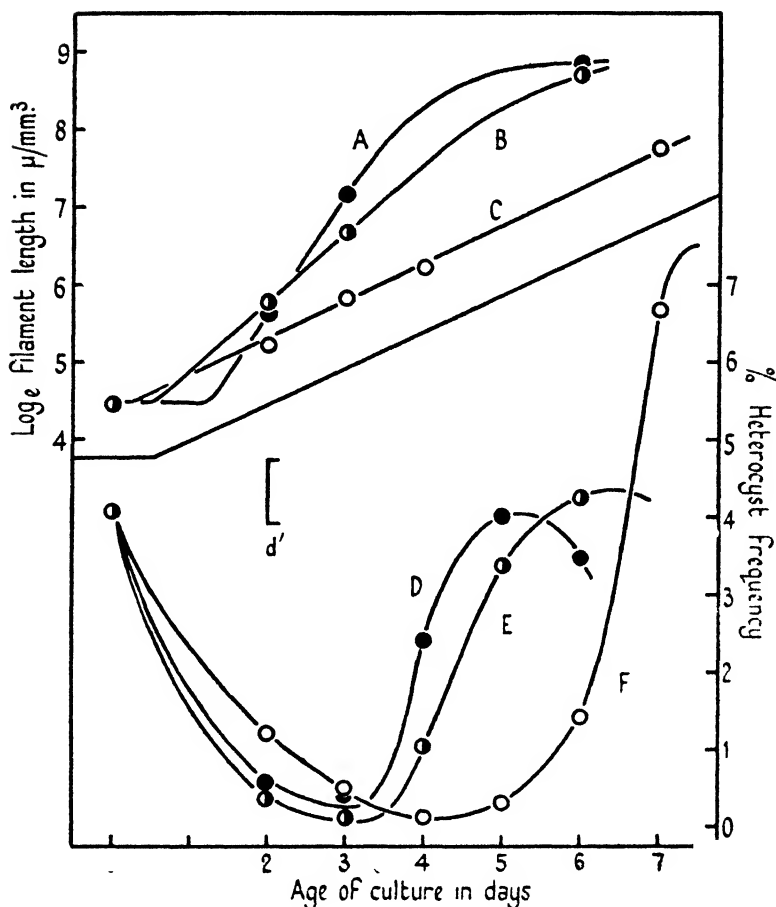


FIG. 7. Effects of different light intensities on growth and heterocyst production in *Anabaena cylindrica*. A, B, C, growth as log_e filament length μ/mm.³ of culture medium; D, E, F, heterocyst frequencies; ○, 260 metre-candles; ◐, 1,300 metre-candles; ●, 3,100 metre-candles; each point the mean of two determinations; d', significant difference at $P = 0.05$ level between two values of heterocyst frequency.

Fig. 7, growth during the exponential period was more rapid, but the lag phase was longer the higher the light intensity. The duration of the lag phase and its standard deviation in each case has been calculated from the regression equation for growth during the exponential period. The growth characteristics are given in the following table:

TABLE I

Light intensity (metre-candles).	No. of determinations used.	Lag period (days).	R.	Duration of exponential growth (days).
260	9	0.245 ± 0.114	0.482 ± 0.014	> 7
1,300	4	0.578 ± 0.432	0.906 ± 0.197	c. 3
3,100	4	1.270 ± 0.157	1.566 ± 0.185	c. 2

Following are tests of the statistical significance of the differences between these. Comparing $R_{1,300}$ and R_{260} the difference is barely significant, $t_{[9]} = 2.14$, $P < 0.1 > 0.05$. Comparing $R_{3,100}$ and R_{260} , $t_{[9]} = 5.84$, $P < 0.001$. Comparing $R_{3,100}$ and $R_{1,300}$, $t_{[4]} = 2.43$, $P < 0.1 > 0.05$. Comparing the length of the lag at 3,100 metre-candles with that at 260 metre-candles, $t_{[9]} = 5.28$, $P < 0.001$. No information has been obtained to indicate the nature of the

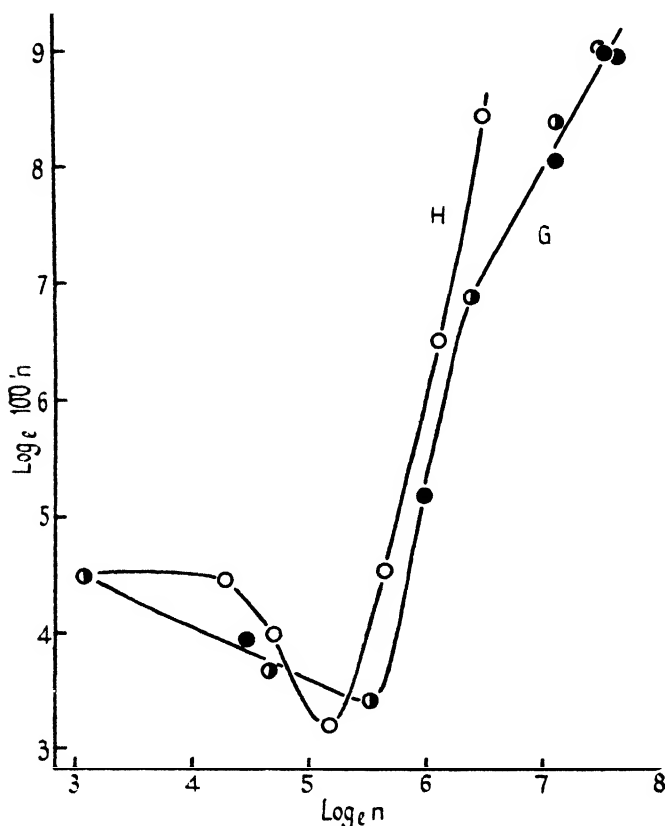


FIG. 8. Effects of different light intensities on heterocyst production in *Anabaena cylindrica*. H, ○, 260 metre-candles, G, ◐, 1,300 metre-candles and, ●, 3,100 metre-candles.

mechanism whereby high light intensities increase the length of the lag period.

In the lowest light intensity (curve *F*, Fig. 7) heterocyst production was delayed in time but the frequency rose to a high level towards the end of the experimental period. The curve for medium light intensity (*E*) is also displaced to the right relative to that for high light intensity (*D*). These differences between the curves are statistically significant. Analysis of the data by double logarithmic plotting (Fig. 8) shows that k apparently had a negative value in the early stages of cultures in all three light intensities. This was probably due to shedding of heterocysts (see p. 255) resulting from a particular

condition of the inoculum and is of no special significance for the present purposes. The points relating to cultures in the two higher light intensities lie very nearly on the same curve and it therefore seems that heterocyst production is not affected by variation in light intensity from 1,300 to 3,100 metre-candles under the experimental conditions used. Comparing the curve for the lowest light intensity (H) with the other (G), the important points are these: (1) the preliminary phase when k is negative is curtailed, (2) during the second phase k has about the same value, i.e. 4, as at higher light intensities, but (3) this phase is prolonged. The first two effects have been confirmed in another experiment carried out at 25° C. using light intensities of 215 and 1,700 metre-candles. The third effect was not clearly shown, but the results obtained do not exclude the possibility of its existence. This second experiment also shows that eventually the curve for the low light intensity becomes parallel to that for higher light intensities.

Experiments with older cultures. To supplement the information obtained using young cultures, two experiments have been made with older material. In these the substances the effects of which were being examined were added to the medium, not before inoculation, but as sterile solution after a period of growth. Determinations of heterocyst frequency and filament length were then made on duplicate sample cultures on three consecutive days and compared with those in control cultures to which only an equivalent quantity of distilled water had been added. Details of the first experiment were as follows: 50-ml. basal medium per culture, additions to give (1) 0.028 M glucose, (2) 0.001 M ammonium chloride; cultures 16 days old with $c.$ 45,000 μ /mm.³ *Anabaena*; temp., 15.5–28.5° C., light intensity, 2,150 metre-candles. During the period of the experiment growth was not perceptible, R , based on 20 determinations of filament length from all three culture series, being 0.005 ± 0.039 , a value which does not differ significantly from zero ($t_{18} = 0.13$, $P \sim 0.9$). Changes in heterocyst frequency seemed best examined by calculation of its regression on time in days for each series of cultures. The regression equations are

Control	$Y = 3.50 - 0.017(x - 1.5)$	$s_a = 0.113$	$s_b = 0.101$
Ammonium	$Y = 3.41 - 0.103(x - 1.5)$	$s_a = 0.088$	$s_b = 0.078$
Glucose	$Y = 3.38 - 0.021(x - 1.5)$	$s_a = 0.142$	$s_b = 0.127$

The regression coefficients do not differ significantly from zero or from each other, e.g. comparing the extreme values, those for the control and the ammonium series, $t_{12} = 0.67$, $P \sim 0.5$, $d' = 8$ per cent. of the mean heterocyst frequency of the control for the ammonium series.

Details of the second experiment were as follows: 50 ml. basal medium per culture, additions to give (1) 0.00025 M succinate, (2) 0.00025 asparaginate; cultures 12 days old with $c.$ 40,000 μ /mm.³ *Anabaena*; temp., 11–21° C.; light intensity 2,150 metre-candles. In this experiment a statistically significant amount of growth occurred. R , based on 20 determinations of filament length from all three culture series, was 0.102 ± 0.029 ($t_{18} = 3.52$, $P < 0.01$). The regression equations for change in heterocyst frequency were

Control	.	.	$Y = 3.88 + 0.226(x - 1.5)$	$s_a = 0.154$	$s_b = 0.138$
Succinate	.	.	$Y = 3.92 - 0.061(x - 1.5)$	$s_a = 0.129$	$s_b = 0.115$
Asparaginate	.	.	$Y = 3.60 - 0.197(x - 1.5)$	$s_a = 0.102$	$s_b = 0.091$

Here the addition of asparaginate has resulted in a definite decrease in heterocyst frequency. Comparing the regression coefficients for the control and asparaginate series, $t_{[12]} = 2.66$, $P \sim 0.02$. Calculation of k shows that the addition of asparaginate has reduced it from 1.54, the value for the control series, to 0.42. The difference between the succinate and control series is not statistically significant, $t_{[12]} = 1.6$, $P \sim 0.1$.

These results show that substances which influence heterocyst production only do so when growth is taking place. Ammonium nitrogen has a clear-cut effect in inhibiting formation of these structures in actively growing cultures but no perceptible effect on heterocysts or normal cells in which growth has ceased. The effect of asparaginate in reducing heterocyst frequency in the second of these experiments was evidently made possible by the slow growth taking place in the cultures.

DISCUSSION

Changes in heterocyst numbers may be brought about in two ways: (1) by the formation of new heterocysts from normal cells, (2) by the germination of heterocysts to give normal cells. Germination of heterocysts has only been observed in one instance (see p. 251). There it was infrequent and its effect on numbers was masked by simultaneous formation of heterocysts. It appears that the changes observed in this investigation are to be attributed to the first of the above causes. Apparent changes in their numbers may be caused by abscission of heterocysts, which are then inconspicuous and liable to be overlooked when counts are made. Abscission may be frequent in old cultures; thus in a 38-day culture 33 per cent. of the heterocysts were detached, but, since in young cultures the filaments are generally long and unbroken, this cause cannot contribute much to the variations observed in this work.

In a previous paper (Fogg, 1944) it has been shown that at different times during the growth of comparable cultures heterocyst frequency is inversely correlated with the total combined nitrogen per unit volume or dry weight of alga. In general agreement with this it has been found in the present investigation that the addition of readily available sources of combined nitrogen to cultures tends to suppress, whereas the supply of assimilable sources of organic carbon tends to increase, heterocyst formation. A direct causative relationship between total nitrogen content and the absence of heterocysts is, however, unlikely. This is shown by some determinations of heterocyst frequency in relation to the total nitrogen content of the alga in culture series grown with different sources of nitrogen. The results, each the mean of 3 determinations on 30-day cultures, are shown in Table II, (p. 256). Here there is no inverse correlation of heterocyst frequency with nitrogen content. It therefore seems that heterocyst formation is inhibited by increasing concentrations within the alga of a specific nitrogenous compound or

compounds, the amount of which is sometimes, but not necessarily always, proportional to the total nitrogen content of the alga.

TABLE II

Medium basal +	Heterocyst frequency.	N content as % of dry weight.
No added combined nitrogen . . .	3.89 ± 0.13	8.14 ± 0.31
0.002 M potassium nitrate . . .	3.47 ± 0.17	6.87 ± 0.14
0.002 M ammonium chloride . . .	0.42 ± 0.16	7.10 ± 0.37

The hypothesis that heterocyst formation is dependent on the concentration of some substance, e.g. an intermediate in carbon metabolism, generally inversely proportional in amount to the combined nitrogen, may equally well be advanced and is not incompatible with the other. Since heterocysts contain conspicuous amounts of cellulose, the availability of such substances is undoubtedly an important factor in their growth. However, the evidence at present available indicates that the role of nitrogenous compounds is the more direct one, since substances such as ammonia at low concentrations exert the more marked effects.

From this working hypothesis it is to be expected that a compound containing nitrogen will inhibit heterocyst formation to an extent depending on the readiness with which it is converted by the alga to the inhibiting substance. In the cases of nitrate, glycine, and asparaginate, the inhibition is transient; only in the case of ammonium salts does the suppression continue so long as an appreciable concentration of the substance remains in the medium. This suggests that the active substance is ammonia itself or some substance readily derived from it. The other nitrogenous compounds can yield ammonia and their effect would appear to be due to a temporary excess of this substance accumulating in the alga before its metabolic balance has become adapted to their presence in relatively high concentrations. The effect of these substances is not confined to a particular phase in the growth of *Anabaena* cultures but follows their addition to unadapted growing material, whether this be made before inoculation or later. Since, in all types of cultures except those supplied with ammonium nitrogen, k eventually reaches the same, relatively constant, value of unity, irrespective of the concentration of available combined nitrogen in the medium, it seems that adaptation of the balance of metabolic reactions must tend to maintain a particular concentration of ammonium nitrogen within the cell.

In a similar manner the effects following the addition of glucose or succinate to material previously grown on an inorganic medium may be explained as due to a temporary preponderance of intermediates of carbon metabolism to which ammonium nitrogen may be linked. Consequently, until the balance of metabolic reactions becomes adjusted to their presence, the concentration of ammonium nitrogen falls below its normal level and heterocyst formation increases.

In certain cases the initial effect of a substance is followed by an opposite secondary effect. Thus, in the cases of glycine and asparaginate, the period of inhibition is followed by a stimulation of heterocyst formation so that finally heterocyst numbers become equal to or greater than those in control cultures. This phenomenon appears to be associated with the presence of a carbon residue in the substance concerned, since the inhibition produced by nitrate is followed by a rise in heterocyst production of only normal duration so that final heterocyst numbers are less than those in control cultures. However, it must be borne in mind that adaptation of certain nitrogen-fixing organisms to nitrate is slow compared with that to other sources of nitrogen (Wilson, Hull, and Burris, 1943), and that consequently the cases may not be comparable. A possible explanation of these secondary effects is that adaptation to the carbon and nitrogen portions of the molecules of asparagine or glycine may proceed at different rates. Thus, in the second phase, the accumulation of carbon residues results in the rate of ammonium nitrogen consumption exceeding the supply so that its concentration falls below the normal level and heterocyst production is increased.

The effect of succinic acid may, perhaps, be explicable along somewhat similar lines. In its presence an increased rate of heterocyst production is followed by a phase of rapid production decreased in duration so that final heterocyst numbers are below those in control cultures. At first succinate may reduce the ammonia concentration by providing a carbon residue with which it may be combined. Later, the organism becomes adapted to utilize the substance for general metabolism and this process preponderates for a time, resulting in an increased concentration of ammonium nitrogen. The absence of the second effect in the case of glucose may be related to a slower rate of utilization.

The marked increase in the rate of heterocyst production following exhaustion of ammonium nitrogen from the medium is evidently due to adjustment not being immediate so that the reactions concerned with its utilization continue at a high rate for a time, resulting in an abnormally low concentration of ammonia within the cells.

If decreased light intensity results in a lower rate of carbohydrate production whilst the rate of nitrogen fixation remains unchanged, the observed effects are not in agreement with hypothesis. On the other hand, it appears likely that under conditions of carbohydrate deficiency nitrogen fixation becomes relatively less, as Wilson (1940) has suggested to be the case in symbiotic nitrogen fixation. If this is so, and a reduced concentration of ammonia in the organism results, the increase in heterocyst production at low light intensities is explained.

Preliminary investigations have shown that variations in the concentrations of the mineral constituents of the basal medium may produce effects on heterocyst frequency. In some cases the results can be related to the known function of the metal or radicle in metabolism. Thus an increased amount of phosphate in the medium produces an increase in heterocyst production

similar to that caused by succinate, perhaps because it induces an increased rate of glycolysis and an accumulation of metabolites similar in effect to succinate. Shortage of molybdenum, which is necessary for nitrogen fixation, produces an increase in heterocyst production.

The results obtained in this investigation give little indication of the mechanism whereby ammonium nitrogen suppresses the formation of heterocysts. The fact that methylamine does not have the same marked inhibitory effect as an ammonium salt does, however, indicate that the effect of the latter substance is produced by direct participation in metabolism rather than indirectly by its effect on intracellular hydrogen-ion concentration. Methylamine, being a base with a dissociation constant of the same order and towards which plant cells have about the same permeability (Pojärvi, 1928), would presumably produce similar changes in the intracellular reaction of *Anabaena* as does ammonia.

A further point that emerges from this study is that the substances found to affect heterocyst formation do so only when growth is taking place. It appears, therefore, that for a given cell to become a heterocyst the concentration of the inhibiting substance within it must not exceed a particular level at some critical phase during its development. In *Anabaena*, in which growth is diffuse but in which the cells of a filament at any particular time are in different stages of division, periodic concentration gradients of the inhibiting substance presumably arise along the length of the filament and heterocysts are formed at the points of lowest concentration. When the mean concentration of the inhibiting substance in the alga is low, such points are more frequent; when it is high, either because of internal causes dependent on the stage of growth or because ammonium nitrogen is supplied from without, they are infrequent.

SUMMARY

1. Experiments are described in which have been investigated the effects of various substances and of different light intensities on the course of growth and heterocyst production in cultures of *Anabaena cylindrica* Lemm.

2. The differential growth factor of heterocyst numbers with respect to total number of cells has been used as a measure of heterocyst production by means of which valid comparisons may be made between different cultures.

3. Substances providing a readily available source of combined nitrogen inhibit heterocyst production when they are added to the medium in which *Anabaena* is grown. In the cases of nitrate, glycine, and asparagine, the inhibition is transient, but ammonium salts produce an inhibition which lasts so long as an appreciable concentration of the substance remains in the medium.

4. Substances such as glucose or succinic acid, which provide an assimilable source of organic carbon, produce a transient increase in heterocyst production when added to the medium.

5. The substances found to affect heterocyst formation only do so when growth is taking place.

6. Heterocyst production is increased in cultures grown in low light intensities.

7. These results are discussed in relation to a hypothesis which supposes the formation of a heterocyst from a normal cell to occur when the concentration within it of a specific nitrogenous inhibiting substance, probably ammonia or some simple derivative of ammonia, falls below a critical level.

My thanks are due to Miss J. P. Roberts for her assistance in carrying out the experimental work on which this paper is based.

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Physiological Studies on Nodule Formation

II. The Influence of Delayed Inoculation on the Rate of Nodulation in Red Clover

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With ten Figures in the Text

INTRODUCTION

IN the introductory paper of this series (Nutman, 1948) a study was made of the rates of nodule and lateral root formation on plants of red clover which had been selected for low and high nodule number. It was concluded from the results that infection takes place at definite points on the root determined by hereditary internal factors, and that these predetermined foci of infection are distributed in the root in the same manner as initials of lateral roots.

In the course of the work it was observed that delay in inoculation led to an apparent raising of the actual rate of nodulation—a result of sufficient interest to warrant confirmation and further investigation.

On the view that random infection of the root occurs so that the number of infections is determined by the virulence of the bacteria, it might be supposed that delay in inoculation, by permitting a larger root system to develop before nodules start to form, occasions a larger surface area of root exposed to infection and that this would result in a higher initial rate of infection. This hypothesis alone cannot account, however, for the fact that (1) the total number of nodules formed after a delay in inoculation exceeds the total number formed upon plants inoculated immediately upon germination, and (2) that a higher rate of nodule formation after a delay in inoculation is maintained. Delay in inoculation will eventually lead on the N-free medium used to nitrogen shortage in the seedlings which will tend to reduce the rate of development of the root system.

The hypothesis put forward in the previous paper, namely, that the bacteria only penetrate the root cortex at certain points and that these foci of infection are identical with the points at which lateral root formation normally occurs, provides a more promising possibility. Thus one may suppose that if inoculation is delayed, the lateral root initials which would normally be converted into nodules may now grow out as roots bearing their own series of foci of infection, some of which would after delayed inoculation proceed to form nodules. In this way the potential number of available foci of infection would

be increased, and provided no factor, such as progressive nitrogen starvation, intervenes to cause a serious retardation in root growth, there is no reason to suppose that the increased rate of nodulation should not be maintained.

This view suggests further consequences of delay which could not be observed in the original experiment, i.e. that there should be an optimum time of delay after which progressive N-starvation of the seedlings leads to retardation of both root and nodule development. It is to the examination of this question that the following work is directed, with the primary aim of throwing further light on the infection processes. In addition, a comparison has been made of the influence of differing periods of delay on nodulation by effective and ineffective strains of bacteria.

MATERIALS AND METHODS

The experimental plants were grown in test-tubes on slopes of an agar medium containing mineral salts but lacking combined nitrogen (Thornton, 1930), as described in the first paper of this series. Single families, or a small number of families of plants, distributed uniformly between treatments, were used in each experiment to minimize variations of a genetical nature. In all experiments care was taken to standardize the volume of the medium in each culture tube (Nutman, 1945). Inoculation was made from a heavy suspension of the bacteria, the two strains employed being strain A, an effective strain, and strain HKC, an ineffective strain, also previously described.

The experiments on the effect of delayed inoculation will be considered in the order in which they were performed, and although they overlap to some extent, the full results for each experiment will be presented. In this way confirmatory evidence on some of the more important effects can be given.

EXPERIMENTAL

Experiment I

In the original experiments the increased rate of nodulation following delayed inoculation was determined from harvest data only, the control (initially inoculated) plants having been harvested at an earlier date than the delayed inoculated plants, so that the results observed might have conceivably been due simply to particularly favourable conditions for nodulation occurring after the harvesting of the control set. It was therefore of first importance to repeat this experiment under conditions such that seasonal effects could be eliminated, and this was done by running side by side two series of treatments; in one series (series A) all the plants were sown together (on March 11) but inoculated at different times, whereas in the other (series B) the 'delay' and control treatments were sown at different times but inoculated at the same time (June 3).

Four periods of delay were used: 20, 40, 60, and 80 days. The two series were overlapped in such a way as to provide a common set which was inoculated after a delay of 80 days (on June 3). Six different families of plants were employed, distributed uniformly throughout the treatments, and at each

sampling date one plant from each family and from each treatment was removed at random for the determination of the numbers of nodules and lateral roots. In addition, counts were made at intervals of roots and nodules by direct observation without taking the plants from the tubes. This could only be done up to about six weeks from sowing, i.e. until the crowding of the roots made accurate counts impossible. These supplementary results are not given in the following tables but are inserted in the figures. The results appear in Tables I and II, and Figs. 1, 2 and 3.

TABLE I

Mean Number of Nodules on Plants inoculated at Different Stages of Growth

SERIES A. Common sowing date.					
Days from sowing (age of plants).					
In parentheses, days from inoculation.					
Interval between sowing and inoculation in days	0	20	40	60	80
	101 (101) 17.6	131 (131) 24.3	154 (154) 21.8	182 (182) 34.8	214 days (214) 36.5
	(81) 42.5	(111) 30.4	(134) 39.7	(162) 41.7	(194) 35.8
	(61) 36.0	(91) 35.8	(114) 33.8	(142) 58.7	(174) 44.6
	(41) 13.2	(71) 14.3	(94) 22.3	(122) 35.7	(154) 35.8
	(21) 7.0	(51) 9.6	(74) 12.0	(102) 23.8	(134) 26.4
SERIES B. Common inoculation date.					
Days from inoculation.					
In parentheses, days from sowing.					
Interval between sowing and inoculation in days	0	20	40	60	80
	21 (21) 0.3	51 (51) 20.7	74 (74) 22.0	102 (102) 38.3	134 days (134) 37.8
	(41) 25.7	(71) 49.2	(94) 46.6	(122) 59.1	(154) 75.6
	(61) 21.3	(91) 20.0	(114) 25.3	(142) 33.4	(174) 35.8
	(81) 13.0	(111) 13.0	(134) 12.0	(162) 18.1	(194) 20.7
	(101) 7.0	(131) 9.6	(154) 12.0	(182) 23.8	(214) 26.4

Note: The maximum number of nodules found at each sampling time are in heavy type.

The tables comprise two sections: series A for the plants with a common sowing date; series B for those with a common inoculation date. In series A

the columns are headed with the number of days from sowing at which sampling was carried out. The figures in brackets denote the number of days between inoculation and sampling times. In series B the columns are headed by the number of days from inoculation at which samples were taken, and the figures in brackets denote the age of the plants (days from sowing) at the time of sampling.

Nodule formation

Series A. Reading down the columns in Table I it is seen that the number of nodules formed in equal times from sowing reach a maximum after 20 to 40 days' delay in inoculation. As the figures in brackets show, the number of days from inoculation to sampling decrease in descending the column; this shorter duration available for nodule formation is, however, not the cause of the appearance of a maximum; for if the entries are read diagonally from the top left-hand corner of the table, which reduces the discrepancy in time for nodule development, the same tendency towards a maximum appears.

Series B. This part of Table I refers to plants inoculated on the same date; and the figures in the columns show the number of nodules formed in equal times after inoculation. Descending the columns, the age of the plants differs as shown by the numbers enclosed in brackets. The great advantage of this series is that all the nodules have been produced under the same external conditions, which is not the case in series A, and variability due to external factors is thus avoided. The set of plants with the 20 days' delay in inoculation now show a maximum nodule number over the whole range of samples. Reading from the top right-hand corner diagonally across the table reduces the age differences in the plants at sampling but shows again equally clearly the optimal effect with 20 days' delay.

Reading along the rows in Table I shows the course of increase in nodule number in the various sets. In series A the values are irregular; the data in series B are somewhat better. Comparing plants of the same age in the two series which have suffered the same delay in inoculation (i.e. comparing entries in series B with numbers in brackets similar to the column headings in series A), large differences in nodule number are seen which may be ascribed to the external conditions during nodule formation. This is suggested by the fact that the discrepancies between the series are chiefly in the control and 20 days' delay treatments, series B giving the larger values. In these sets the growth of the plants in series A took place mainly in the spring and in series B in midsummer. Temperature has been shown (unpublished data) to have a marked effect on nodulation rate, and the effect of low temperature is also commented upon in a following experiment.

The rate of nodule formation can be better judged from the graphs in Figs. 1 and 2 which are constructed from the data of series B. In Fig. 1 the nodule number is plotted against the number of days from inoculation; in Fig. 2 against the logarithm of time.

In the figures the data in Table I are supplemented by those derived from

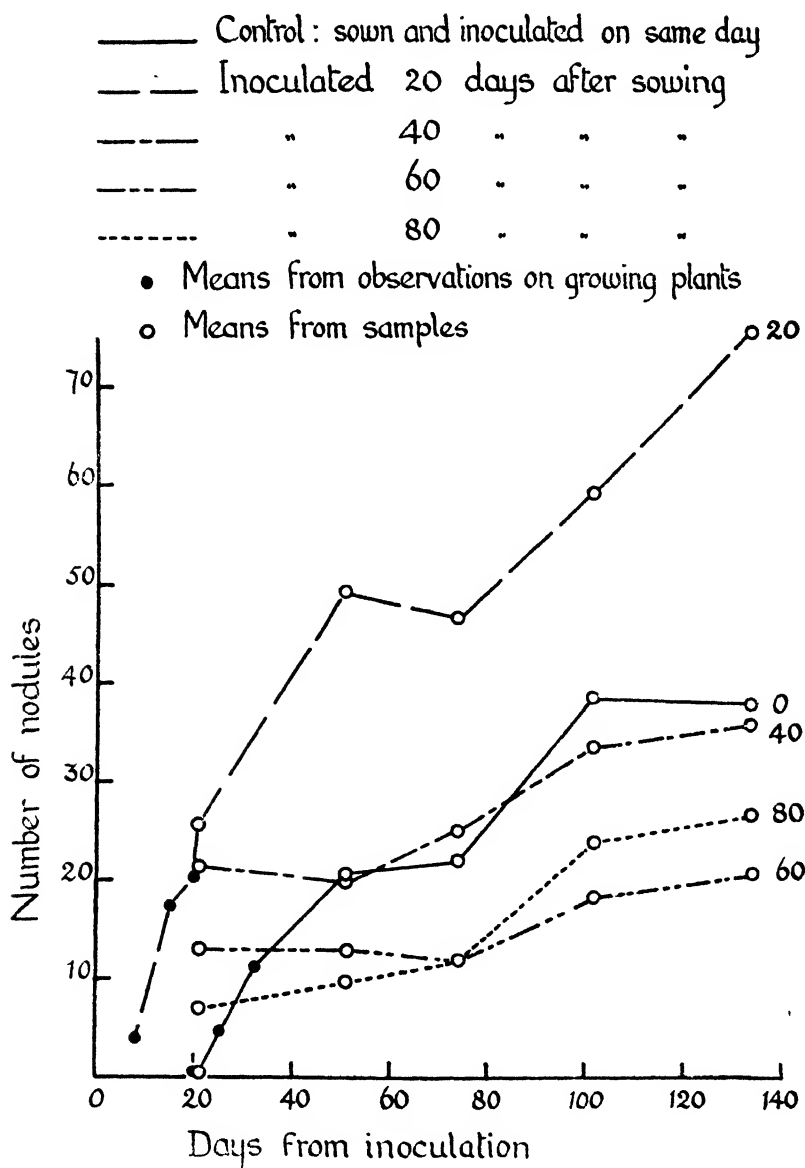


FIG. 1. Mean no. of nodules on plants grown for 0, 20, 40, 60, and 80 days before inoculation with the effective strain A. All sets inoculated on same day—series B.

nodule counts made on plants in the tubes. The curves in Fig. 1 fall into two groups; the curves for delay of 0 and 20 days run an approximately parallel course at different levels; the curves for sets with delay of greater than 20 days show a marked discontinuity round about 80 days from inoculation with an arrest in nodule formation before this time and a rate of nodulation similar to that in the controls after this time. These relations are seen

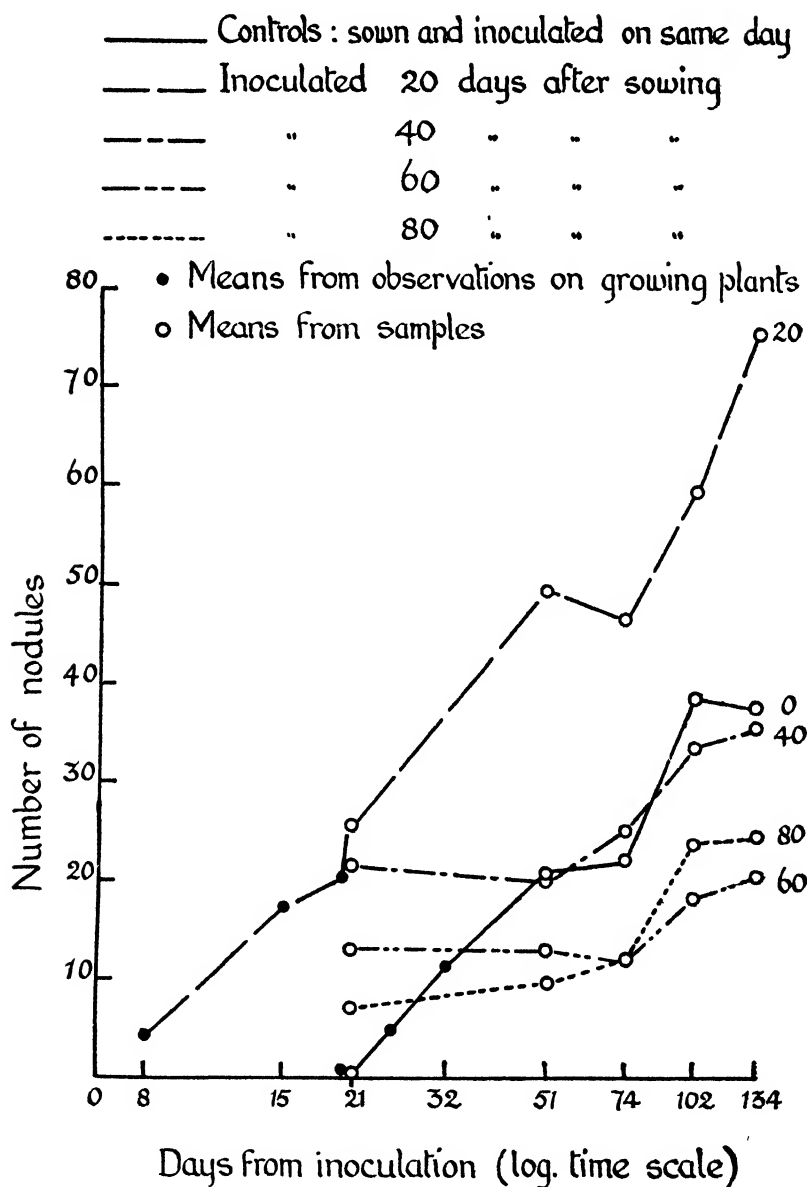


FIG. 2. Mean no. of nodules on plants grown for 0, 20, 40, 60, and 80 days before inoculation with the effective strain A. All sets inoculated on same day—series B.

more clearly in Fig. 2, in which logarithmic plotting is used. In the control and 20-day delay sets the relation of nodule number to the logarithm of days from inoculation closely approach straight lines (Nutman, 1948) and these lines are sensibly parallel. The arrest of nodulation in the other sets is clearly seen and also the sudden onset of nodulation to a rate similar to that in the control set.

Thus the higher level of nodulation following a delay in inoculation of 20 days is due to an earlier start in nodule formation as compared with the control set, or alternatively, the lag period in nodule formation of 3 weeks in the control set is reduced in the 'delayed' set to some 6 days. It is well known that the invasion of the roots by the nodule organism is controlled by some diffusible substance originating in the plant (Thornton, 1930), and during the delay period this substance is formed and therefore earlier nodulation follows delayed inoculation. The data in Fig. 2 shows that such an earlier formation of nodules occurs also in the series with delayed inoculation of more than 20 days. Thus 21 days after inoculation the number of nodules in the 40-day delayed set is the same as in the 20-day set; longer delay in inoculation presumably again retards nodule formation, though even 80 days' delay still results in some nodules after 21 days, the lag period in the controls. Data on the earliest stages of nodule formation in the longer delayed sets are not to hand, and therefore it cannot be decided whether the lower nodule number seen at 21 days after increasing period of delay in inoculation is due to lower rate of nodule formation or to an earlier arrest. There can be no doubt from the data in Fig. 2 that in the sets with greater than 20 days' delay an arrest of nodule formation occurs. This arrest is related to a cessation at the same time of lateral root production.

Lateral root formation

The data for number of lateral roots formed are entered in Table II and number of laterals plotted against the logarithm of the age of plants are shown in Fig. 3. These data are for plants sown on the same day (series A) and the sampling data are supplemented by direct counts of roots of the seedlings in the tubes so long as counts of this kind were possible.

It is evident that up to 45 days from sowing the rate of lateral formation is the same for all the series with delayed inoculation and these exceed the root number of the control set inoculated at sowing. This confirms the results previously reported (Nutman, 1948) and has been accounted for by the conversion of potential root meristems into nodules in the control set.

The linear relation of root number to logarithm time indicates that the absolute rate of increase of roots falls continuously. After 45 days the control set and the 20-day delayed set show an increase in the rate of lateral formation, and the lead in root formation in the delayed set is maintained for the next 60 days. The longer the delay in inoculation the lower the rate of lateral formation during this period, so that after 100 days the number of laterals falls conspicuously in passing from the 20 to the 80 days' delay sets. This lag in root production is doubtless related to nitrogen starvation of the seedlings which cannot be relieved until inoculation has taken place; the longer inoculation is delayed the greater will the nitrogen deficit become.

In all the sets as time progresses the rate of lateral formation increases, and the 'delayed' sets tend to catch up the others. Since the days from sowing used to construct the diagram include the pre-inoculation time, the later

portions of the curves obscure this point. The values in series B of Table II, however, in which root number is related to days from inoculation, show this process of levelling up quite clearly. It seems fairly certain therefore that the

TABLE II
Mean Number of Lateral Roots on Plants inoculated at Different Stages of Growth

SERIES A. Common sowing date.					
Days from sowing (age of plants).					
In parentheses, days from inoculation.					
Interval between sowing and inoculation in days	0	20	40	60	80
	101 (101) 29.5	131 (131) 38.0	154 (154) 89.0	182 (182) 78.3	214 days (214) 53.8
	(81) 36.8	(111) 42.4	(134) 76.0	(162) 76.7	(194) 90.8
	(61) 21.3	(91) 29.8	(114) 58.0	(142) 77.5	(174) 62.3
	(41) 20.8	(71) 25.3	(94) 38.3	(122) 45.6	(154) 52.8
	(21) 15.5	(51) 24.8	(74) 25.6	(102) 40.0	(134) 44.0
SERIES B. Common inoculation date.					
Days from inoculation.					
In parentheses, days from sowing.					
Interval between sowing and inoculation in days	0	20	40	60	80
	21 (21) 6.2	51 (51) 16.6	74 (74) 22.8	102 (102) 35.8	134 days (134) 44.8
	(41) 18.0	(71) 19.1	(94) 31.3	(122) 49.4	(154) 48.2
	(61) 23.3	(91) 19.6	(114) 22.3	(142) 55.0	(174) 54.5
	(81) 21.3	(111) 18.1	(134) 30.7	(162) 45.1	(194) 41.0
	(101) 15.5	(131) 24.8	(154) 25.6	(182) 40.0	(214) 44.0

divergence of the curves in Fig. 3 is due to temporary starvation of nitrogen, and that as nitrogen fixation is established the rate of lateral formation becomes independent of the original delay in inoculation.

The treatment with delay of inoculation of 20 days formed nodules before N-starvation set in and the larger number of secondary roots formed afforded by their growth an increase in both nodules and lateral roots during the second phase of lateral root development. An optimum in both nodule and lateral root formation occurs therefore in this set.

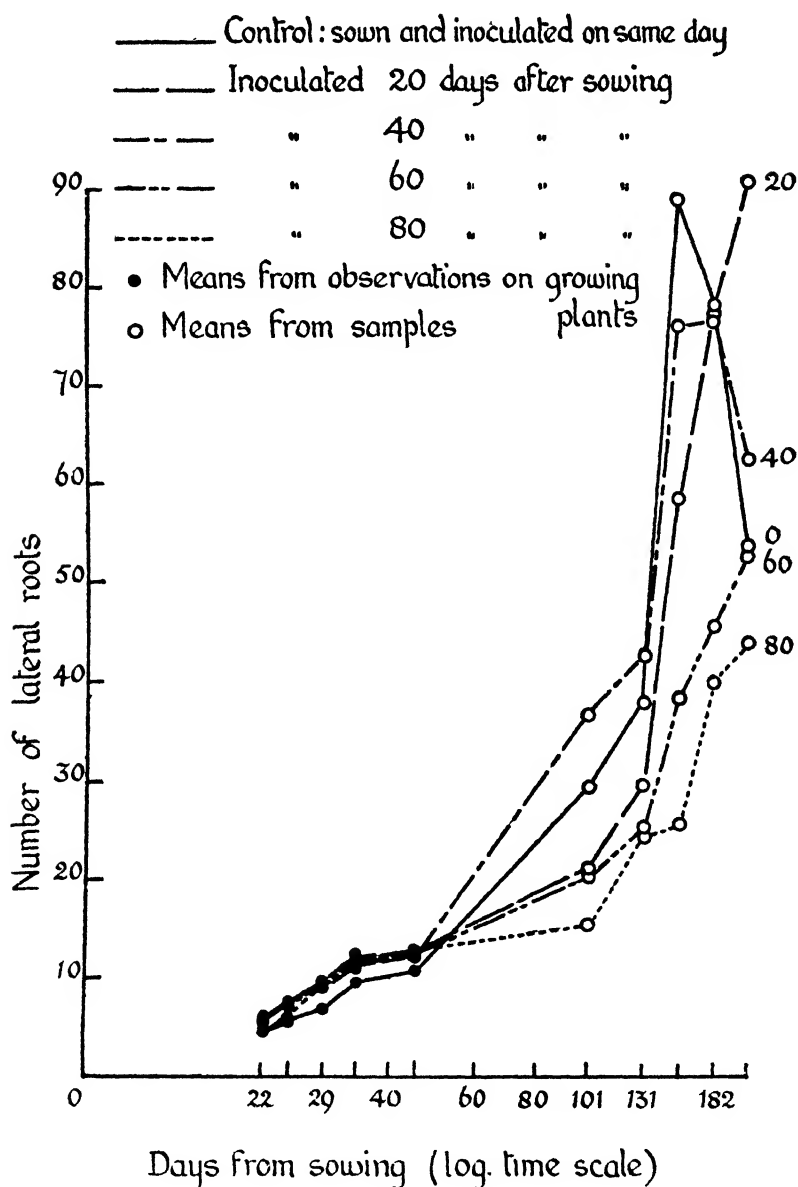


FIG. 3. Mean no. of lateral roots on plants grown for 0, 20, 40, 60, and 80 days before inoculation with the effective strain A. All sets sown on same day—series A.

Experiment 2

In experiment 1 both the control and delayed sets were inoculated with an effective strain of nodule bacteria, and it appeared to be of some interest, in view of the known differences in nodulation of effective and ineffective strains, to determine the rate of nodulation with ineffective strains following delayed inoculation.

This experiment consisted of four treatments only, i.e. control *v.* delay, and effective strain inoculation (strain A) *v.* ineffective strain inoculation (strain HKC). Several families of known genetic constitution were used, the treatments being distributed uniformly amongst them and the samples for counting removed at random as in the first experiment. The delayed treatments were sown before the controls, both sets being inoculated on the same day. The experiment was begun in January 1947 and owing to the exceptionally long period of cold and overcast weather during which the temperature in the greenhouse fell,¹ the period of delay was extended to 31 days, the plants having then reached about the same stage of development as at 20 days in the previous experiments.

The results are presented in Fig. 4, and it is at once evident that, with respect to delay, effective and ineffective strain inoculation show different results. With the effective strain delay has resulted in an enhanced rate of nodule formation, as in the first experiment; while, with the ineffective strain, nodule formation starts earlier with respect to the time of inoculation and there is a transient increase in rate of nodule formation lasting some 5 days, after which no difference in rate is seen in this experiment. A direct comparison of the rates may be made over parts of the curves approximating to straight lines (i.e. excluding the final observations with strain A and the earlier observations with strain HKC) with the following results. With strain A inoculated at sowing the mean daily rate is 0.78 nodules and after delayed inoculation 1.26 nodules per day, whereas with ineffective strain inoculation the rates are 0.92 and 0.89 nodules per day respectively.

The divergence in the behaviour of the strains does not appear to be due to the nitrogen-fixing ability of the effective strain, since it occurs from an early stage when both sets were independent of fixed nitrogen.

A further result of some interest in the experiment is the effect of the long period of low temperature. This has very much slowed down the production of nodules during the first part of the experiment. As a result the rate of nodule formation at the very earliest stages has been more closely studied than heretofore, and it appears that there is at first, for about 1 week, no difference between the rates of formation due to strain differences, and in both strains delayed inoculation leads to higher rates.

The main conclusion to be drawn from this experiment is that delayed inoculation has a very transient effect with the ineffective strain as contrasted with the effective strain in which delay leads to maintained increase in the rate of nodule formation.

Experiment 3

This experiment was designed to investigate further the strain differences noted above, and to examine more fully the effects of varying periods of delay in inoculation. In other respects the setting up of this experiment

¹ This was a consequence of the fuel shortage during this period.

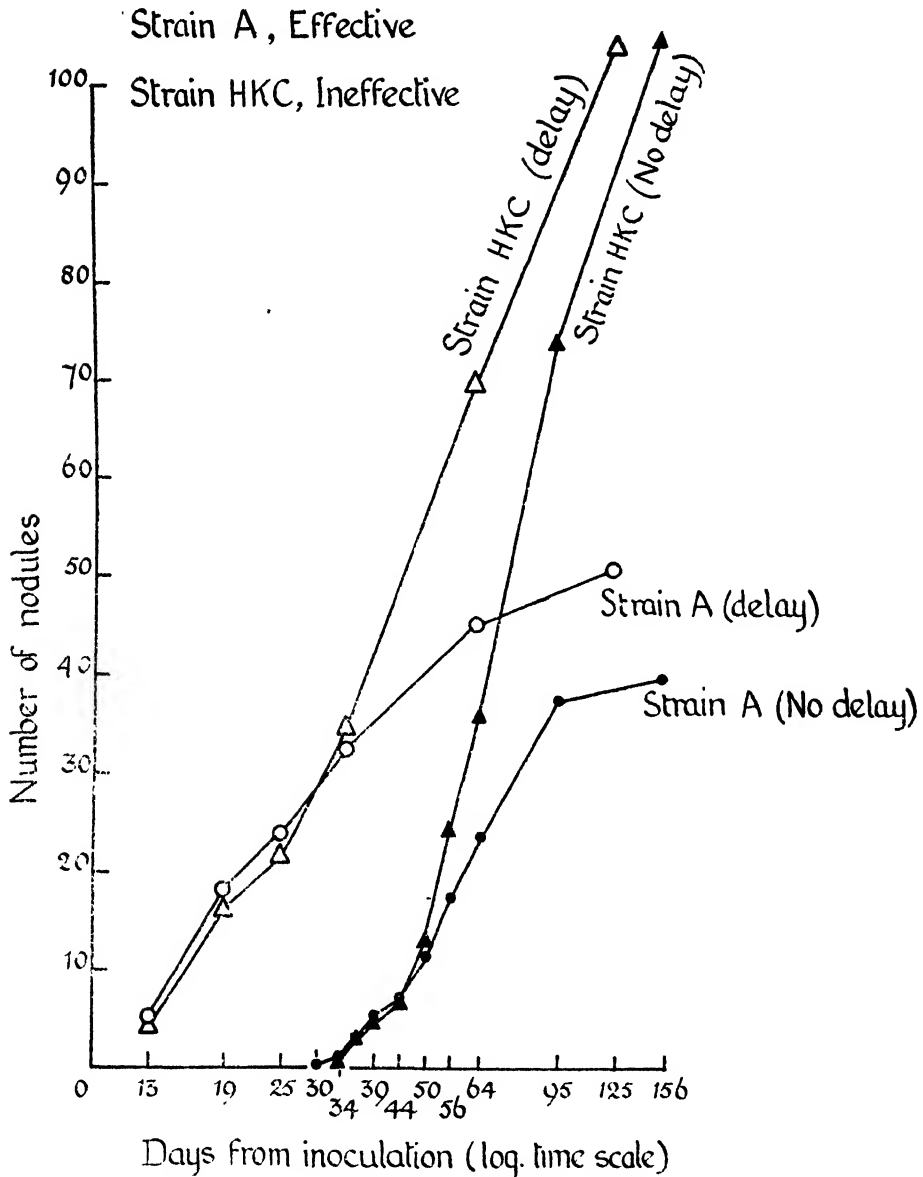


FIG. 4 Nodule formation following inoculation at sowing and after a delay of 31 days with effective and ineffective strains of bacteria.

was similar to the foregoing. The strains used were A. and HKC, and a common inoculation date (July 2) was employed. The periods of delay were 0, 6, 12, 18, 23, 31, and 41 days. The results are presented in Figs. 5-6 with the same scales of reference as used in experiments 1 and 2 (lateral root counts were not made).

The strain differences noted in experiment 2 are confirmed, and in addition

it is seen from Fig. 5 that the maximum rate of nodulation by an effective strain follows from 18 to 23 days' delay. Even 12 days' delay appears to give a slight increase in rate, although nodules do not start to form on control plants until about 3 days afterwards.

In the earlier stages of the experiment the effect of increasing delay in inoculation consistently leads to an increase in the number of nodules formed

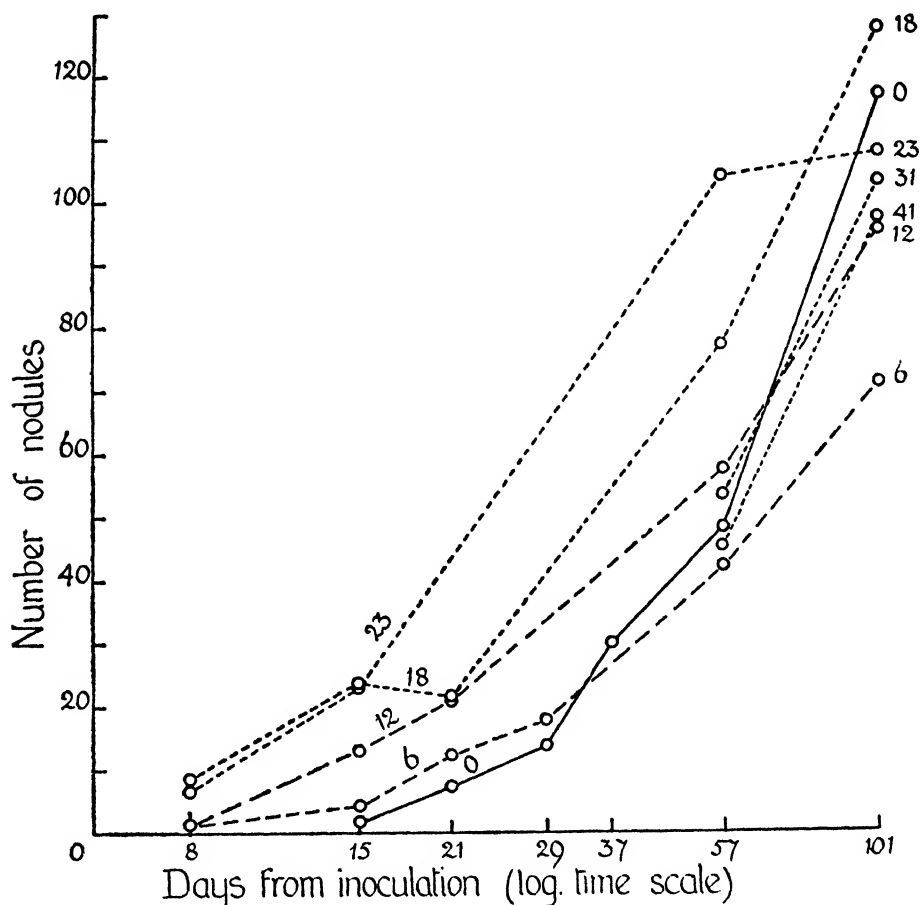


FIG. 5. Nodule formation following inoculation with the effective strain A at sowing and after a delay of 6, 12, 18, 23, 31, and 41 days from sowing.

up to that time, but in the later samples the differences are no longer so marked. Thus the control set at the last sample gives a nodule number far greater than that for 6 days' delay; the irregularity in the order of the sets at the last sample is due to the increasing variability in the nodule numbers on the individual plants as the experiment proceeds. Judging from the course of the curves as a whole, there is good evidence that the maximum nodulation follows a delay of 18–23 days and that further delay reduces the nodule number. Delay of 6–12 days shows at first a higher rate of nodulation, but this

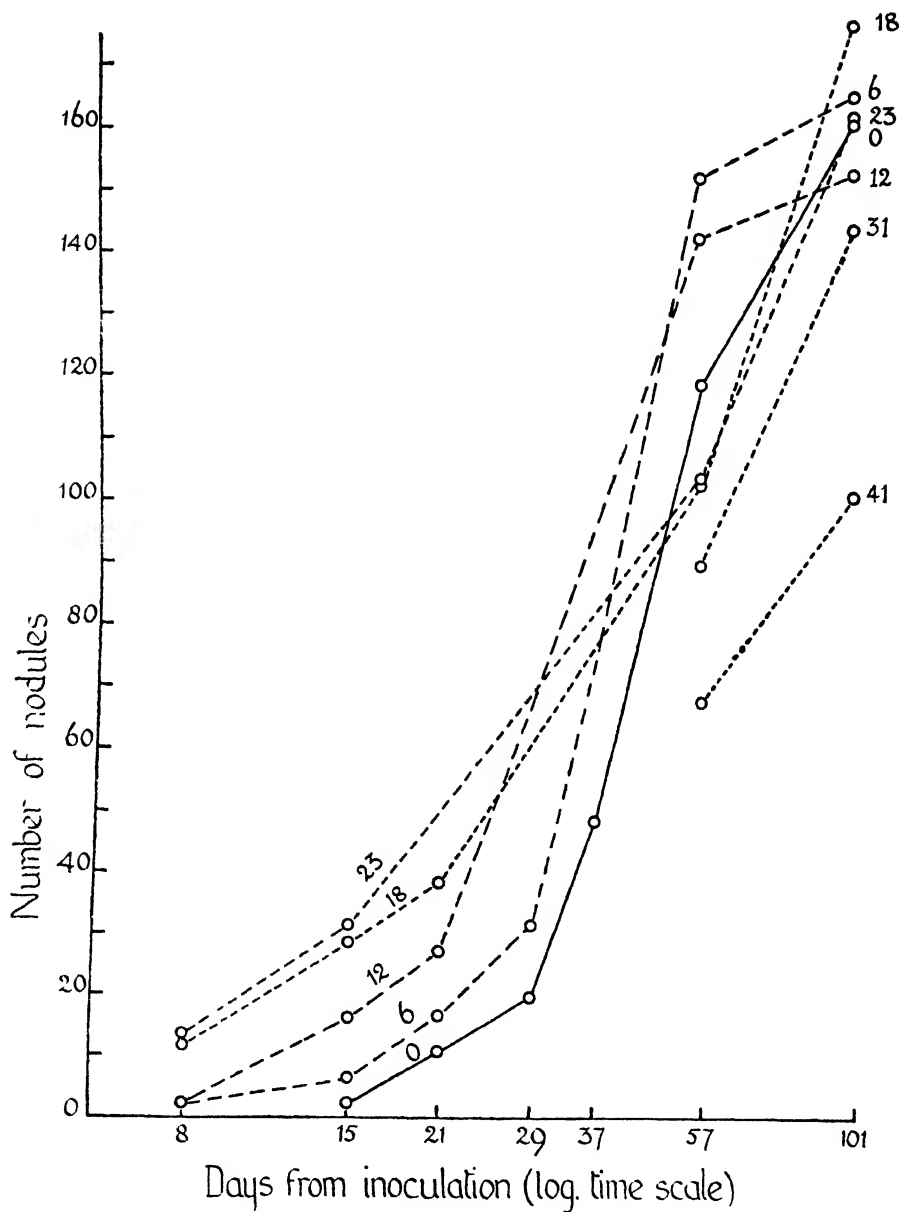


FIG. 6. Nodule formation following inoculation with the ineffective strain HKC at sowing and after a delay of 6, 12, 18, 23, 31, and 41 days from sowing.

effect tends to disappear. In the various sets the lag period in nodule formation progressively diminishes from the control set through the intermediate sets and is least in the series with 18–23 days' delay. With regard to the strain differences (Figs. 5 and 6), which are reflected not only in the final

numbers of nodules formed but also in the rates of formation, it is seen that with effective strain inoculation the curves showing rates are all more or less parallel, but with ineffective strain inoculation there is a consistent tendency for the mean rates to fall off with increasing delay in inoculation. This result may be brought out more clearly by comparison of actual rates over the congruent portions of the curves, i.e. between 15 and 57 days after inoculation in Fig. 5 and between 29 and 57 days in Fig. 6. With effective strain inoculation the daily rates of nodule formation are respectively in the control and delayed sets 1.11, 0.91, 1.06, 1.25, and 1.94 nodules, and with ineffective strain inoculation the corresponding rates are 3.53, 4.31, 2.79, 1.55, and 1.27 nodules per day. Thus with effective strain inoculation the mean rate remains at about the same level in the control and 6 and 12 days' delay sets and shows a tendency to rise after 18 and 23 days' delay, but with ineffective strain inoculation there is progressive falling off in rate as the period of pre-inoculation growth increases.

It is worth noting that after an initial delay in inoculation of about 20 days the number of nodules formed by an effective strain approach those produced by an ineffective strain inoculated immediately upon sowing, the actual figures being 125 for the delayed effective and 160 for the ineffective without delay.

DISCUSSION

The aim of this investigation on the physiology of nodulation is to analyse the factors concerned in determining the number and site of nodules in the clover plant. In the previous paper an explanation of the limitation of the number of nodules on the root was offered in terms of a physiological homology between roots and nodules, and it was also suggested that inhibitory factors may be concerned in their development. The present paper provides data which may be interpreted in similar terms, and the dependent hypothesis, namely, that inhibitory substances are concerned, has been further developed and extended to explain the supposed differing virulence of strains.

A cursory consideration of this problem discloses the fact that two major aspects are concerned: (1) the morphogenic factors controlling the development of the root system, and (2) the factors contributed by the nodule in such control of root development and the interaction between these two sets of factors.

1. The orderly development of the root system suggests that co-ordinating factors are at work, though little has been added to our knowledge on this matter since the pioneer work of Sachs. Thus it is by no means certain that a dominance action of the primary root meristem occurs comparable with that seen in the shoot of many plants. Incidental observations by Thimann (1936) and van Overbeek (1939) on this point are not adequate to establish such dominance with any certainty. For the shoot the apical dominance has been attributed to the action of auxins arising in the apex which are conducted down the shoot and exert an inhibiting effect on lateral buds. There seems

little doubt that the root apex can also produce auxins (Cholodny, 1924, 1933; Keeble, Nelson, and Snow, 1931; Hawker, 1932; Boysen Jensen, 1933), and that the geotropic response of the root is related to this auxin production. This, however, does not in any way compel the conclusion that inhibition of lateral roots is under the control of the apical meristem, and with even less certainty can it be stated that the initiation of lateral roots is affected by the auxin production by the apex of the main root. In general the plant auxins exert an inhibitory effect on root extension, though claims have been made that at very high dilution root extension is accelerated by auxins (Thimann, 1936).

The very large body of work on the culture of isolated root-tips has shown that an extraneous supply of auxin is not necessary for the maintenance of root development, so that it would appear that in the development of the root, so far as supply of auxin is concerned, this organ is autonomous and not dependent on the shoot. The nature of the nodule bacteria, however, is also concerned in this aspect of the problem, since it has been shown that active cultures produced β -indolylacetic acid (Thimann, 1936a; Chen, 1938), and there is some evidence that the effective strains produce less than the ineffective (Georgi and Beguin, 1939).

2. The larger number of nodules produced by ineffective strains as compared with effective might be accounted for on various grounds, and a discussion of this aspect would be incomplete without reference to the carbohydrate and nitrogen content of the plant. It is well known that any modification of the environment of the plant which tends to alter the C/N ratio, such as, on the one hand, addition of nitrogen to the culture medium or shading of the leaves, or, on the other hand, increasing the carbon dioxide content of the air, increasing the light intensity, or inoculation with ineffective strains, also alters the amount of nodulation. In this connexion it may be noted that delay in inoculation also results in a rise in the C/N ratio and an increase in nodulation.

Wilson (1935) and Wilson and Fred (1939) have put forward on the basis of this correlation a general hypothesis that there is a causal relationship between the C/N ratio and the number of nodules formed on the root. On this view the high rate of nodulation by ineffective strains is due to the low nitrogen status of the inoculated plants. This is not in accordance with the results in this paper, since a difference in the rates with effective and ineffective strains is apparent with seedlings equally nitrogen starved.

Without the postulation of a mechanism it is difficult to assess the value of a very simple chemical hypothesis of this kind. It is clear that the addition, for instance, of combined nitrogen to the root medium has manifold effects and the reduction in nodulation (by effective strains) which follows may be more directly ascribed to the striking reduction in the proportion of curled root-hairs (Thornton, 1936) or to morphogenic effects. The whole question will be more fully discussed in a later paper dealing with the effect of added nitrogen on lateral root formation and nodulation.

In the previous paper evidence was presented to show that nodules and

lateral roots originate from the same meristematic centres, that these are limited in number and predetermined in position. The question then arises as to the casual mechanism determining the preponderance of nodule formation in the ineffective strains. Within the limits set by the number of these foci of development, which is a genetical factor concerned with the plant only, the much smaller proportion occupied by nodules with the effective strains may be due to a varying inhibition exerted by pre-existent nodules on initiation of subsequent module development, this inhibition being greater with effective strains of bacteria. An alternative possibility is that nodules occupied by ineffective strains have a promoting effect on further development. It has been shown that in the very early stages of growth of the seedling nodule formation occurs equally rapidly with the two strains. Two deductions may be made: (a) there is no promoting effect at this stage by ineffective nodules on subsequent nodule development, for otherwise the ineffective strain should from the start show a higher rate of production; and (b) the intrinsic virulence of the bacterial strains is not a discriminating factor in the rate of nodulation.

The later nodule formation with the ineffective strain is much more rapid. The evidence on this point presented in the first paper of this series has thus been corroborated. The most probable explanation for this well-attested fact is that in the very early stages of nodule development occasioned by these two strains no mutual effect of the nodules occurs, but that later, when nodule development has proceeded, the effective nodules have some inhibitory action which is absent in the ineffective nodules. This may be related to the meristematic development in the nodule itself, for in ineffective nodules meristematic activity is arrested, whereas in effective nodules it proceeds for a longer period (Chen and Thornton, 1940). The varying size of the nodules is a result of this differential behaviour of the meristem. In this connexion it may be stated also that within each type of response there is an overall inverse correlation between the number of nodules produced and their average size; this point is illustrated in Figs. 7 and 8.

In an extreme case an ineffective symbiosis may even be associated with the formation of very few abnormally large nodules; in these, however, the bacterial tissue has degenerated and the nodule is a hollow structure, while the meristem continues to function. This relationship in ineffective symbiosis also suggests that the source of the inhibitory activity is in the apical meristem rather than in the bacterial tissue of the nodule.

If inhibitory substances are released during meristematic activity and are translocated about the root, a mechanism of control would thus be provided. Experimental methods for the investigation of this point may be cited; thus excision of nodules in the early stages should lead to a more rapid development of further nodules in the effective strains, but should have little or no effect in the ineffective. Preliminary work along these lines has been begun and the results to date support the hypothesis. It might be anticipated, in view of the fact that nodule and lateral roots are equivalent, that the root apex is itself

capable of producing similar inhibition. The fact that the apex of the root is never occupied by bacteria rather supports this contention, but experimental evidence is required. Preliminary work has shown that the roots of clover seedlings produce a substance that has an inhibitory effect on nodulation, but the location of this substance is still unknown (Nutman, 1945).

Effective Strain A

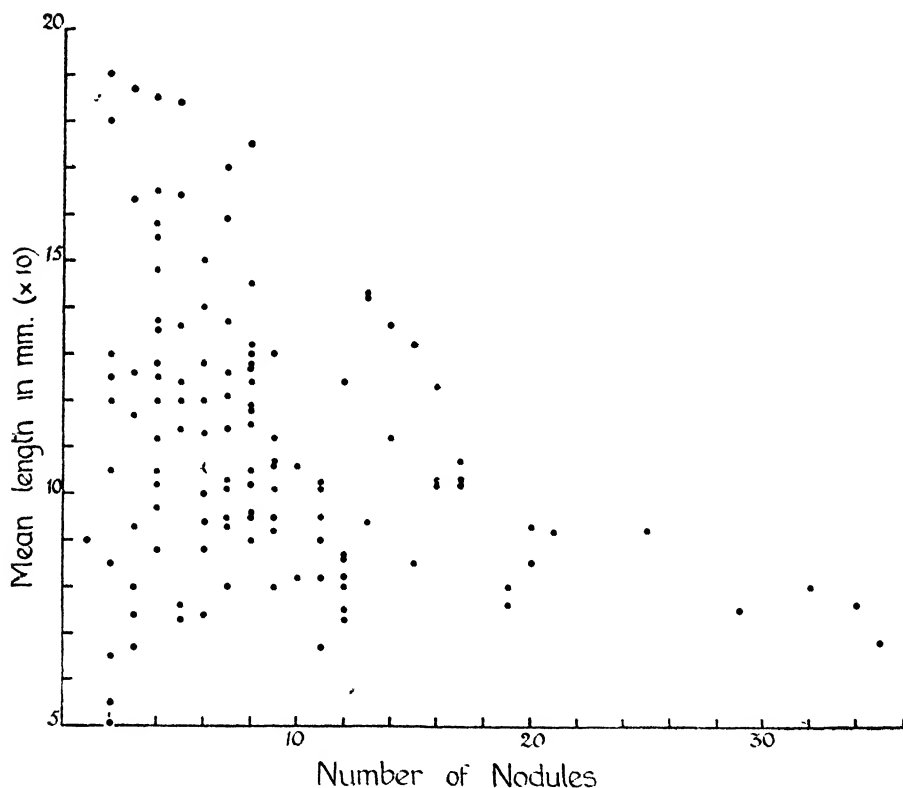


FIG. 7. Relation of nodule length and nodule number in individual plants inoculated with effective strain A.

The immediate aim of this paper is to analyse the effect of delayed inoculation on subsequent nodulation. The following results may be taken as established: (1) delayed inoculation reduces the time-lag between inoculation and the appearance of the first nodules on the root. This effect increases progressively up to approximately 20 days' delay and can be fully accounted for by the excretion from the root of substances favouring invasion of the bacteria (Thornton, 1930). This substance is produced by the cotyledons and leaves and is independent of nodule activity. Delayed inoculation thus in no way interferes with this process. (2) Longer delay than 20 days does not prevent

this preliminary formation of nodules (Fig. 1), but it is followed by an arrest of nodule formation in the case of the effective strain of bacteria. This arrest is associated with a cessation in lateral root production. It seems unlikely that

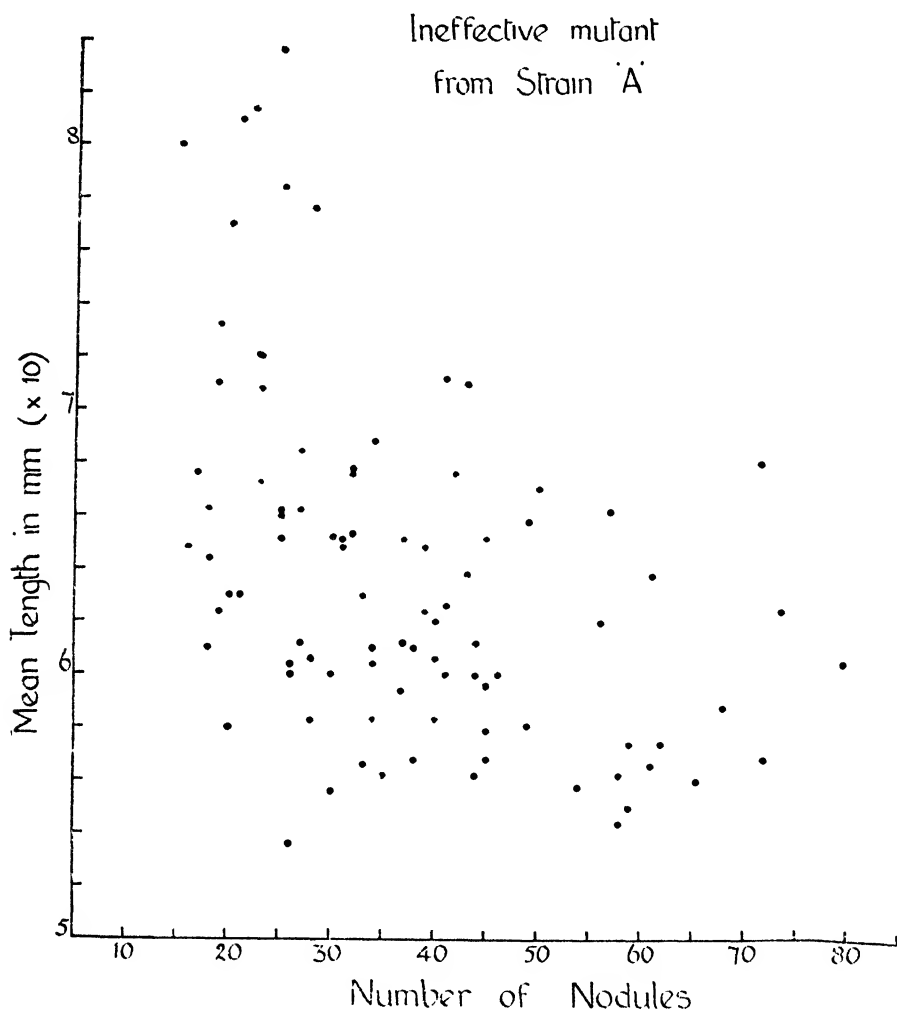


FIG. 8. Relation of nodule length and nodule number in individual plants inoculated with ineffective mutant from strain A.

nitrogen starvation is the sole factor concerned in this arrest of nodulation, for if this were the only cause one would expect that this arrest would be greatest with the 80-day delay inoculation and progressively less with shorter delay.

An examination of Fig. 3 shows that this is the case so far as lateral root formation is concerned, but on the other hand, as the data in Fig. 2 show, arrest in nodulation is as severe in all cases of delay from 40 to 80 days in spite

of the fact that the nodule numbers at 21 days from inoculation are very different. Nitrogen starvation, however, is likely to have effects upon the shoot also, and indeed observation showed that the longer the delayed inoculation the fewer the leaves on the plants, and at 100 days from sowing (series A) the mean leaf numbers observed were as follows: in control 9.6; delay of 20 days, 12.4; 40 days, 9.1; 60 days, 8.2; 80 days, 6.6. The leaf numbers thus reflect very accurately the number of nodules seen at this time. That leaf activity plays some part in nodule formation is almost certain to be the case (Nicol, 1934). It may therefore be concluded that the arrest in nodulation following long delay is purely nutritional in origin. As the nitrogen status of the plant is re-established, nodulation proceeds at comparable rates in all sets.

The position with regard to the ineffective strain is different, as the results in Figs. 4 and 6 show. With the ineffective strain the rate of nodulation falls progressively with delay in inoculation and this is no doubt due to the incapacity of the nodules to provide sufficient nitrogen. Attention has been drawn to the different course in lateral root production with effective and ineffective strains in Fig. 4 of the previous paper. The nitrogen and other nutrient requirements for the production of ineffective nodules and of lateral roots is very different. Whereas the nodules are organs of determinate growth and usually remain very small with the ineffective strain, lateral roots are, on the other hand, indeterminate in growth and require further supplies of nutrients for their continued development. It is therefore possible for continued formation of nodules to proceed by the use of the available nitrogen in the plant, whereas this would not be possible for continued root production.

In conclusion it may be worth while to review and interpret as far as is at present possible the whole process of infection in terms of root morphogenesis, and in the diagram (Fig. 9) an attempt has been made to distinguish and illustrate its various stages. Fig. 10 illustrates the above explanation of the contrasting effect of delayed inoculation with effective and ineffective strains.

In the first stage the growth of the bacteria outside the root leads to the production of a secretion, which has been shown to have the properties of indolylacetic acid (Thimann, 1936*a*) and which causes root-hair deformation. A proportion of the curled root-hairs are next infected and the bacteria develop within the root-hair the characteristic callose enclosed infection thread (McCoy, 1932). In the third stage a few of these root-hair infection threads grow into the cortex and the formation of the nodule follows. A thorough restudy of the course of nodule formation in the pea has recently been published by Bond (1948) and shows the same sequence of events as in clover. The penetration of the infection thread has been considered here and in the previous paper to take place only in the neighbourhood of incipient meristematic activity, and has been shown (Wipf and Cooper, 1940) to be associated with polyploid cells in the root. An infected root-hair has in all probability only a limited period of life, and it is well known that formation of nodules on the older parts of the root system does not take place. Thus infection is limited to the zones A in the diagram on the younger part of the

root, and particularly to the points F lying within these zones which can be regarded as indeterminate incipient meristems.

The number of nodules appearing on the root will depend upon the number of the points F arising within zone A and upon their conversion into

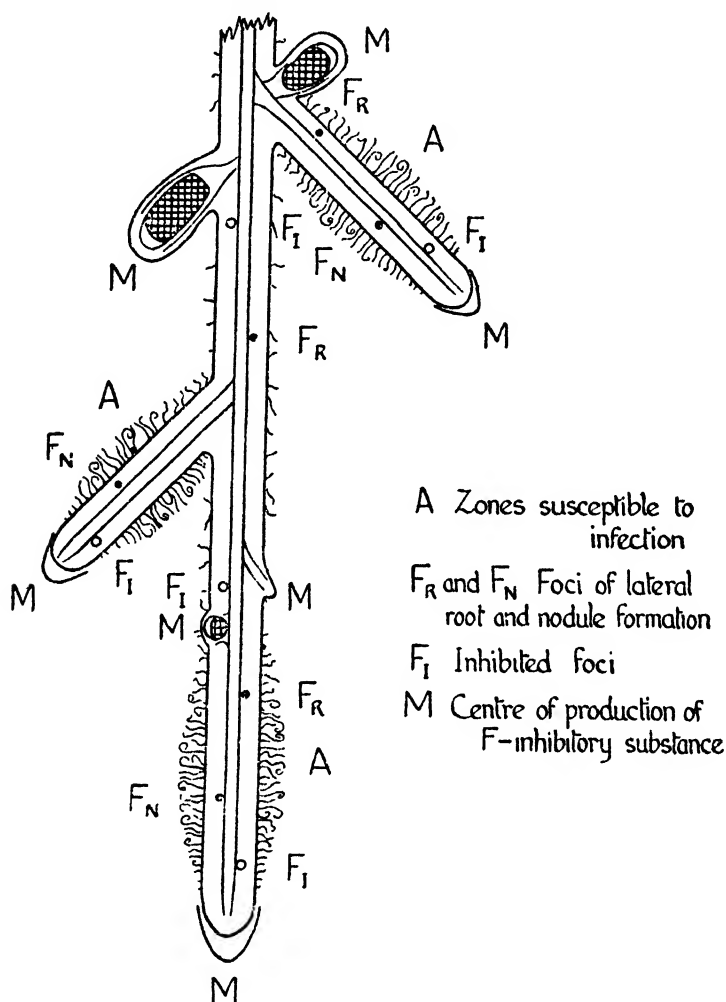
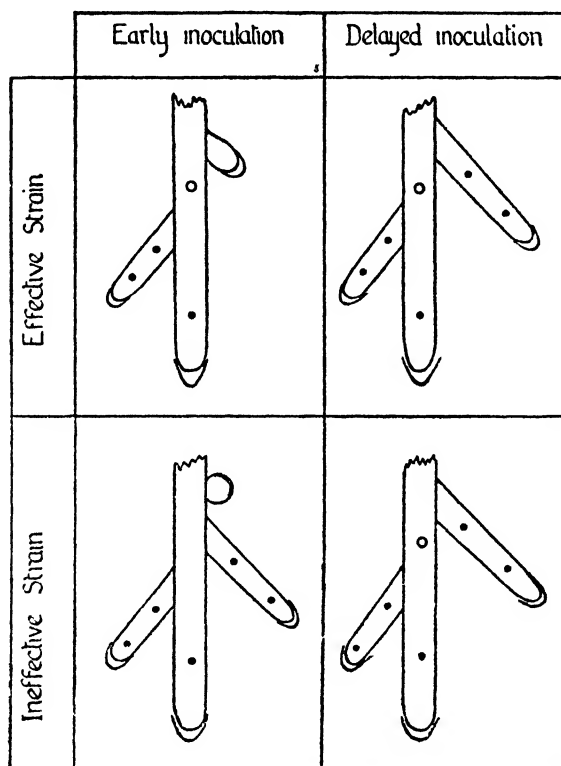


FIG. 9. Diagram to illustrate the manner in which sites of nodule and lateral root development may be determined

nodule rudiments which, with any given set of external factors, is determined by (1) the genetic constitution of the plant, and (2) the production of inhibitory substances in the root and nodule apices (M). It is still a matter of uncertainty whether complete suppression of these incipient meristems (F_I) can be caused by apical control. These considerations account for the independent genetic control of nodule number and the relationships between laterals and nodules. Strain differences can then be explained by one further assumption, namely,

that the amount of inhibitor produced is related to the activity of the meristem, a root apex producing as much or more of the inhibitory substance than the meristem of an effective nodule, and an ineffective nodule meristem producing an inappreciable quantity.

The outstanding fact to be elucidated is thus the higher rate of nodule formation in the ineffective strains as compared with the effective in spite



Key: ○ Inhibited meristematic foci
• Foci of Nodule formation

FIG. 10. Diagram illustrating effects of immediate and delayed inoculation with effective and ineffective strains of bacteria upon root and nodule development.

of the incapacity of the ineffective strains to fix nitrogen. To account for this, emphasis has been placed upon an hypothesis of inhibition of further nodules with effective strains by pre-existent nodules, an action which is supposedly associated with the meristematic zone in the nodules and shared also by the meristems of the root. The justification for the postulation of hypothesis in biology has been very cogently presented by Woodger (1948) and the heuristic value of such a procedure lies in the suggestion of crucial experiments. It will be the aim of further work to substantiate this hypothesis.

SUMMARY

1. Nodule formation in red clover, grown on agar slopes in test-tubes and inoculated at sowing, begins at about the $2\frac{1}{2}$ -leaf stage of development, i.e. at about 15 days, and at a progressively falling rate. Relative to the logarithm of the age of the plant this rate is constant.

2. In the initial stages of nodule formation (i.e. during the first few days) no differences appear in the rate for effective and ineffective strains, but subsequently ineffective strain inoculation leads to the formation of about twice as many nodules as effective strain inoculation.

3. With the effective strain of bacteria delay in inoculation of from about 12 to about 30 days after sowing leads to (1) a reduction in the lag period between inoculation and the formation of the first nodules, and (2) to an increase in the rate of nodule formation which is maintained.

4. Longer periods of delay with effective strain inoculation lead to nitrogen starvation in the seedling, and arrest in leaf and root development accompanied by a progressive reduction in the rate of nodule formation. Following delay in inoculation of more than 3 weeks, nodule development takes place in three distinct stages: (i) a moderately rapid phase, (ii) a period during which nodule formation is completely arrested, (iii) resumption of nodule formation at a reduced absolute rate.

5. With an ineffective strain of bacteria, except for a transient increase during the first week, delay in inoculation is followed by a decrease in rate of nodule formation.

6. An interpretation of these results is put forward in terms of root morphogenesis. It is suggested that bacteria penetrate the root and produce nodules only within those zones of the root distinguished by the presence of growing root-hairs and only at points of incipient meristematic activity (i.e. foci of lateral root formation). Development at these foci is considered to be determined by an inhibiting activity of the meristems (lateral root and nodule) already present. Thus normal nodule formation leads to a lowering of the meristematic potentiality of the root by the metamorphosis of lateral meristems into nodules which are not self-reproducing, whereas delay in inoculation allows a larger actively reproducing meristematic system to develop which has thus a higher capacity for infection.

It is further suggested that with ineffective strain inoculation this limiting effect is not evident because the ephemeral nodule meristem produces no appreciable amount of the inhibitor.

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Observations on the Anatomy of *Durvillea antarctica* (*Chamisso*) *Hariot*

BY

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With Plates VI and VII and eight Figures in the Text

DURVILLEA is a giant seaweed of antarctic and subantarctic waters, where, on account of its large size, it forms a conspicuous member of the algal population. When fully grown the plant may attain a length of 10 metres, the massive fronds being 'almost too heavy for a man to lift' (Hooker, 1847).

The thallus of *Durvillea* consists of an attaching disc and a short, cylindrical stipe, which becomes flattened at its upper end and passes into an elongated leathery lamina composed of a varying number of segments.

Two principal species of *Durvillea* are usually recognized—*D. antarctica* and *D. Harveyi*—differing principally in the form of the lamina, which is much more dissected in the former than in the latter. That of *D. antarctica* consists of numerous strap-like segments and closely resembles a laminarian in form. That of *D. Harveyi*, on the other hand, is hardly dissected at all, and may consist of a single, entire, and rather broad frond. The fronds of *D. antarctica* are a dark greenish-brown in colour, whilst those of *D. Harveyi* are a lighter, yellowish-brown (Skottsberg, 1921). In addition, the lamina of *D. antarctica* is inflated by a central, honeycomb-like system of air chambers, whilst that of *D. Harveyi* is solid throughout.

In J. B. Hooker's 'Botany of the Antarctic Voyage' a further distinction between the two species is made by means of the attaching organ. That of *D. antarctica* (= *D. utilis* (Bory)) is stated to be circular and entire in contrast with that of *D. Harveyi* which is composed of a number of anastomosing strands similar to the haptera of the Laminariales. Grabendörfer (1885), however, describes only old plants as possessing attaching organs of this nature, and Skottsberg (1907) was able to find only one very large plant with a disc 15 cm. in diameter, which showed any signs of subdivision.

Areschoug (1855) included *D. antarctica* and *D. Harveyi* within a single species. Skottsberg, however, considers typical *D. antarctica* and *D. Harveyi* too widely different to warrant their inclusion within a single species. Specimens showing features intermediate between the two types have been described (Skottsberg, 1921, 1941). This might be explained either by hybridization or by the two 'species' being merely different habitat forms of one and the same species (Skottsberg, 1921, 1941). This latter view is held by

Herriott (1923), who considers it to be further supported by the form of a specimen of *D. antarctica* collected from Bounty Island, a more than usually exposed habitat, and which showed even greater subdivision of the frond than usual.

D. Montagnei (= *Laminaria caepestipes* of Montagne), a form described by Areschoug as being intermediate between *D. Harveyi* and *D. antarctica*, is considered by Skottsberg (1907, 1921) to be a form of *D. Harveyi*. The genus *Sarcophycus* (Labillardière, 1806; de Toni, 1895; Turner, 1819; Whitting, 1893), established in 1847 by Kützinger, is also considered to be a species of *Durvillea* (Harvey, 1863; Skottsberg, 1907).

D. antarctica is an inhabitant of the lower littoral region and extends almost to low-tide level. The stipe grows out horizontally from the rocks, so that at low tide it supports the whole weight of the lamina. Old plants may attain a length of 10 metres, with stipes of a diameter up to 3 in. and discs from 10 to 12 in. across (Herriott, 1923). It always grows in association with *Macrocystis pyrifera*, and its attaching disc often affords shelter for small marine molluscs which burrow into its tissues (Oliver, 1923). *D. Harveyi* favours somewhat more sheltered situations than *D. antarctica*, often growing where the force of the breakers is lessened by a shelf of rock (Skottsberg, 1921). The two species are sometimes found growing together, in which case *D. Harveyi* is usually found at a slightly lower level than *D. antarctica* (Skottsberg, 1941).

D. antarctica is found off the coast of South America from central Chile to Cape Horn, off Kerguelen's Land, the Falklands, the Campbell, Auckland, and Chatham Islands, and off New Zealand. The distribution of *D. Harveyi* is similar, but it has not been reported from New Zealand (Skottsberg, 1921, 1941; Agardh, 1848; de Toni, 1895).

The plant under investigation was collected in New Zealand in 1934, and on its arrival in England¹ it was photographed entire (Pl. VI). It was then halved longitudinally, and one half was rubbed with glycerine to keep it in a supple condition and the remainder was preserved in alcoholic glycerine.

The plant is considered, on account of the numerous whip-like segments of the lamina with the central air chambers, to be a specimen of *D. antarctica*.

The first description of *D. antarctica* was made by Chamisso, under the name of *Fucus antarcticus*. Later, in 1826, Bory, describing the specimen collected during the voyage of Captain Duperrey, founded the genus *Durvillea*, but, ignoring Chamisso's earlier description of the plant, gave it the specific name of *D. utilis*. In 1892 Hariot gave the plant its correct name of *Durvillea antarctica*.²

The first anatomical investigation of the genus was carried out by Graben-dörfer (1885), who worked on material collected from the south Brazilian coast. His account is of *D. Harveyi*, but his identification of the species does not seem at all certain, since he regards the formation of the air chambers, not

¹ The plant was in a living condition on arrival, having been carefully packed and brought by air.

² Quoted by Herriott (1923): no source given.

as a specific difference, but as a feature of adult organization. Hence it is not at all clear that the species under consideration is really *D. Harveyi*. The anatomy of the material he investigated shows many points of similarity with the present investigation of *D. antarctica*.

More recently (1923) Herriott made some observations on *D. antarctica* collected from points off the coast of New Zealand in the region of Christchurch, but she did not describe in any detail the anatomy of the material investigated.

Thus, although *Durvillea* has been known for a considerable length of time, very little information concerning the anatomical structure is available.

EXTERNAL MORPHOLOGY

The plant investigated is a fairly young one, with an overall length of just over 6 ft. (Pl. VI).

The attaching disc is roughly circular in outline, with a diameter of about 2 in. The lower surface of the disc is very irregular where it has been in contact with the rocks, and in places the shells of small marine molluscs are embedded in it. In the middle of the lower surface is a small portion shaped like an inverted cone (Text-fig. 7), the appearance of which suggests that it may have been the original attaching organ of the very young plant, and the expanded portion of the disc may have been formed later, but a study of very much younger plants is necessary to see whether this is the case.¹

The stipe is 8 in. long, with a diameter of just over an inch. It is brown in colour with many transverse cracks, mainly confined to one side.

At its upper end the stipe broadens out into a flattened 'palm' from which arise the main segments of the lamina which become subdivided into numerous whip-like segments. The palm is less thick than the rest of the lamina, which is inflated by a system of central air chambers (Text-fig. 1 A). The segments of the lamina taper towards their tips (Pl. VI), where they are less inflated and are lighter in colour, suggesting that they are actively growing.

The palm, like the stipe, is characterized by numerous small cracks, particularly on one surface, where they are arranged in a number of discontinuous arcs radiating from the top of the stipe.

Herriott (1923) relates the cracks on the stipe to the strains imposed upon it at low tide when it supports, in a horizontal position, the whole weight of the fronds. Consequently cracks tend to develop on the upper surface of the stipe, and, as the stipe bends, the cracks open, thus giving a greater range of bending. As this specimen is a detached one, it is not possible to say whether the cracks are on the upper side, but only that they tend to be developed more on one side than the other.

Scattered everywhere over the surface of the lamina, except at the tips, are the conceptacles, appearing as darker brown spots on the light brown of the lamina and as rather paler spots on the dark brown of the palm.

¹ Since this investigation was completed, some younger plants have arrived from New Zealand, but of these even the smallest independent plants—about 15 mm. in size—already possessed a disc too well developed to see whether this was the case.

ANATOMICAL STRUCTURE

The structure of the apex

Serial sections through the apex—both in the longitudinal and in the transverse direction—reveal neither the presence of an apical groove nor of a specialized apical cell or cells. This is in agreement with the observations of Grabendörfer (1885), who was also unable to find any apical cell.

Median longitudinal sections perpendicular to the plane of flattening show a dome-shaped apex bounded by a layer of small cells, all of which seem to be equally actively dividing (Text-fig. 1 B, *mer.*). The cells of this layer are brick-shaped with rounded outer ends, and the whole outer surface is covered with a layer of deeply staining mucilage.

Cell division appears to be confined to this surface layer, the cells of which divide repeatedly by both periclinal and anticlinal walls (Text-fig. 2 A). The horizontal anticlinal divisions result in growth in length, and the periclinal divisions give rise to the medulla and the cortex. The periclinal divisions of the cells at the extreme tip of the dome give rise to longitudinal files of cells which become stretched by the continued divisions of the surface layer and form the elongated cells of the primary medulla (Text-fig. 1 B *med.*). The cortex is similarly formed by the periclinal divisions of the cells farther from the tip of the dome, and is added to progressively as the distance from the apex increases.

The outer members of the cortex are arranged in files perpendicular to the surface and are slightly elongated in that direction (Text-fig. 2 A), and their relationship to the meristoderm cells from which they are derived can be clearly seen. The repeated horizontal, anticlinal divisions of the meristoderm lead to the longitudinal stretching of the inner members of the cortex which grade into the medulla. Possibly this elongation of the inner members of the cortex adds to the medulla, as is the case in other members of the Fucales.

In the outer regions of the cortex the cells have fairly well-defined walls and form a compact tissue, but the cells of the inner cortex and the medulla are widely separated by a non-staining mucilaginous region, apparently formed by the swelling of the cell-walls.

Hyphae are present right up to the extreme tip of the lamina, interweaving between the cells of the medulla, but none could be seen originating in this region. At the apex, sections cut in the plane perpendicular to the plane of flattening show numerous hyphae cut transversely (Text-fig. 1 B), showing that here they are running horizontally. This, together with the lack of any points of origin, suggests that the hyphae penetrate *upwards* into the actively growing tip from the older tissues behind it. Hyphae are not usually regarded as being present right up to the apex of the lamina of the Fucales. Fritsch (1945a) states that in *Fucus* 'hyphae are only produced 'at a considerable distance behind the apex'. The condition is similar in *Halidrys* and in *Asco-phyllum* (Oltmanns, 1889). Moss (1948) finds, however, that in *Fucus vesiculosus* hyphae are present as near as 4 mm. to the vegetative apices, though

not penetrating to the extreme tip. In the sterile tips of receptacles of *F. vesiculosus* which have lost their apical grooves, however, she finds¹ that hyphae are present right up to the extreme tip, where they run horizontally in a manner similar to that seen in this material of *D. antarctica*.

The development of the mature lamina structure

Immediately behind the apex the thallus consists of three principal regions—meristoderm, cortex, and medulla. The meristoderm consists of a layer of small, brick-shaped cells with dense contents and rounded outer walls. The cortex is about 5 to 6 cells in depth, consisting of fairly regular cells arranged in files perpendicular to the surface, and, by their arrangement, obviously derived from the meristoderm. In this region the meristoderm is repeatedly dividing by horizontal, anticlinal walls, thus bringing about an increase in the length of the lamina (Text-fig. 2 B). The outermost cortical cells are elongated in the direction perpendicular to the surface, whilst the inner ones are slightly elongated in the longitudinal direction and are drawn out into rather irregular shapes. The centre is occupied by a medulla of cells considerably elongated in the longitudinal direction and arranged in uniseriate, longitudinal files separated by non-staining mucilage. A few hyphae are present, interweaving between the cells of the medulla and inner cortex (Text-fig. 1 C).

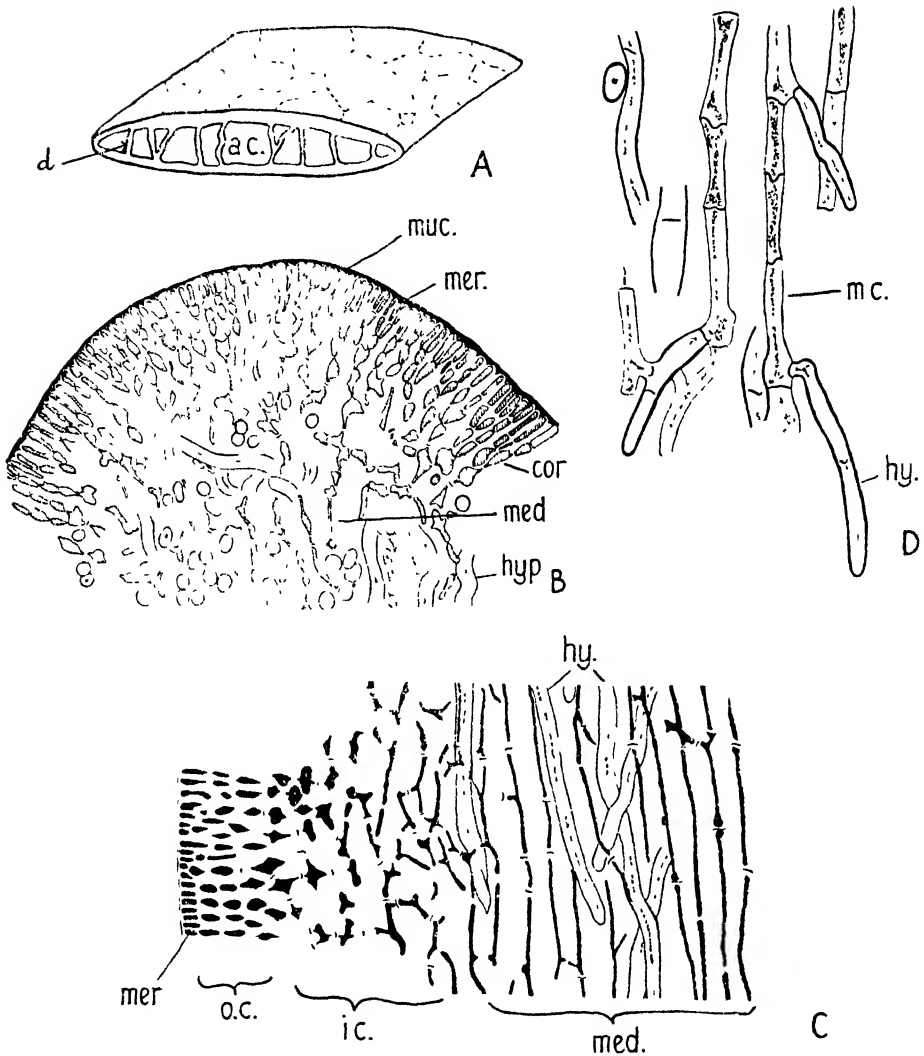
Thus, the youngest portion of the thallus consists of three tissues, medulla, cortex, and meristoderm, similar to the three primary tissues seen in *Fucus* and throughout the Fucales, but the component cells are very much smaller than in *Fucus* (Text-fig. 2 D, E).

As the distance from the apex increases, the lamina becomes both broader and thicker, most of the increase at first being due to the repeated divisions of the meristoderm and to the swelling of the walls. The cells cut off by the periclinal divisions of the meristoderm form an *outer cortex* of cells arranged in files perpendicular to the surface. These cells have very dense, granular contents and may possibly be concerned with assimilation. The cells are slightly elongated in the direction perpendicular to the surface, and as their distance from the exterior—and from the apex—increases, the radial walls become progressively more swollen, and the protoplasts become widely separated from each other. The tangential walls, however, remain sharply defined and highly refractive. This outer cortex increases in extent with increasing distance from the apex. In the mature lamina it is about 10 cells in depth (Text-fig. 2 C); in the solid palm it is from 10 to 20 cells in depth, and in the stipe it may be as many as 40 cells in depth, the number of cells increasing towards the base of the region.

As the distance from the apex increases, the radial walls of the inner cortical cells become extremely swollen and the protoplasts become drawn out into an irregular reticulum (Text-fig. 1 C, *i.c.*).

With increasing distance from the apex the longitudinal walls of the medullary cells also swell and become ill defined, the horizontal walls remaining

¹ Moss, unpublished.



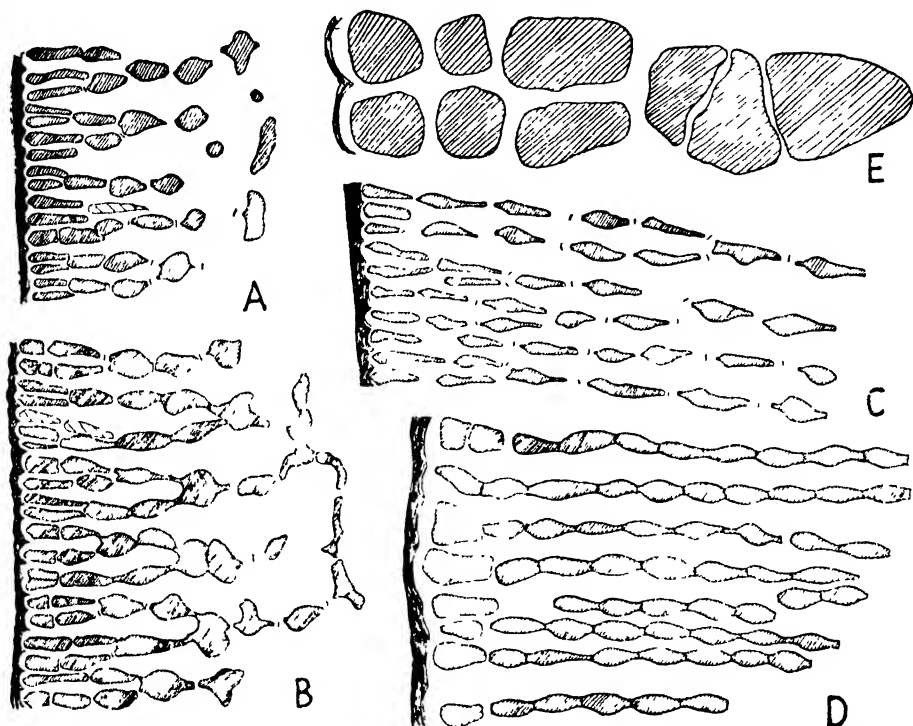
TEXT-FIG 1. A, A portion of the lamina to show the arrangement of the air chambers (*ac*), with the outlines of the air chambers indicated on the upper surface by broken lines. B, Median longitudinal section through the apex of an actively growing tip, showing the meristoderm (*mer.*) and the formation of the cortex (*cor.*) and the medulla (*med.*). *hy.*, hyphae; *muc.*, layer of mucilage. (Scale = $\times 220$) C, Longitudinal section 1 mm behind the apex. *o.c.*, outer cortex, *i.c.*, inner cortex. (Scale = $\times 220$.) D, Longitudinal section about 1 mm behind the apex showing the origin of hyphae from medullary cells (*m.c.*). (Scale = $\times 350$.)

sharply defined and highly refractive. Consequently the protoplasts become separated from each other in the horizontal direction and the medulla appears to consist of longitudinally running uniseriate series of cells.

The formation of hyphae

Hyphae are present right up to the apex, but none were seen originating until a distance of several centimetres behind the apex, where they arise as

outgrowths from the longitudinally elongated medullary cells (Text-fig. 1 D). In this region they were not seen arising from the cortical cells, but in the stipe they were (cf. p. 296 and Text-fig. 5). The hyphae have thick walls, narrow lumina, and only occasional transverse septa. They run principally in the longitudinal direction and are directed both towards and away from the apex.



TEXT-FIG. 2 A series of longitudinal sections of the thallus, A, B, and C in the plane perpendicular to the plane of flattening and D in the radial direction. A, L.S. through the apex, just at the base of the dome. Divisions both anticlinal and periclinal. B, L.S. 1 mm. behind the apex. Very active anticlinal divisions. Correlate with growth in length. C, Mature lamina, outer cortex, part only. Anticlinal divisions less marked than in B. Growth in length here less active. D, R.L.S. stipe, outer region only. Divisions here almost entirely periclinal. Growth in thickness is here more important than growth in length. There is also swelling of the meristoderm cells. E, A few cells from the meristoderm and cortex of the stipe of *Fucus vesiculosus* drawn to the same scale. (Scale = $\times 475$.) N.B. The few cells shaded in the opposite direction are in another plane.

In unstained preparations the hyphae have rather refractive walls, but in preparations stained with alcoholic Gentian violet the hyphal walls stain a rather reddish-purple, and consequently, in the apical regions, are easily distinguishable from the cortical and medullary cells, since their walls do not stain at all with Gentian violet and their contents stain blue. This differential staining reaction was found to be best produced by using a 0.1 per cent. solution of Gentian violet in 50 per cent. alcohol. The staining was done under the microscope, using the minimum amount of stain so that it evaporated almost to dryness during the process. The blue coloration of the cell

contents appeared first, followed, on longer staining, by the redder coloration of the hyphal walls. Immediately the redder colour appeared the excess stain was dried up and the section mounted in 50 per cent. glycerine. With this treatment the differential coloration is fairly permanent, and is still present in sections mounted 18 months ago.

This differential staining probably indicates some difference in the chemical composition of the walls of the hyphae and those of the medullary and cortical cells. The appearance of a reddish coloration with Gentian violet is interpreted (Delf and Hyde, 1936) as indicating the presence of mucilage and a blue colour as an indication of pectin.

Hence, on account of their staining reaction, it is very easy, in the region of the apex, to distinguish between the hyphae and the longitudinally running filaments of medullary cells. Within a few millimetres of the apex, however, a layer becomes differentiated around the protoplasts of the medullary cells also staining a reddish colour with the Gentian violet, so that, farther from the apex, distinction between the medullary cells and the hyphae is very difficult, if not impossible. The hyphae can, however, sometimes be distinguished by their thicker walls, by their less frequent and less sharply defined transverse walls, and by the fact that they stain rather more deeply than the medullary cells.

The formation of the air chambers

The first stage in the formation of the air chambers characteristic of the nature lamina can be seen about 3 cm. behind the actively growing apices.

Small hyphae, similar to the longitudinal ones, but of smaller diameter, arise from the medullary cells in this region and grow out horizontally in the plane perpendicular to the plane of flattening. This causes the medullary cells to become displaced from their longitudinal course, and the longitudinal hyphae become separated into two plates. These small hyphae anastomose freely and fuse with each other and with the longitudinal hyphae and the medullary cells (Text-fig. 3 A), thus producing a complicated network of branches. This fusion of hyphae with other hyphae and with other cells has not been previously recorded amongst the Fucales, but is a process resembling the *initial* stages of the formation of secondary pit connexions in the Laminariales (Killian, 1911).

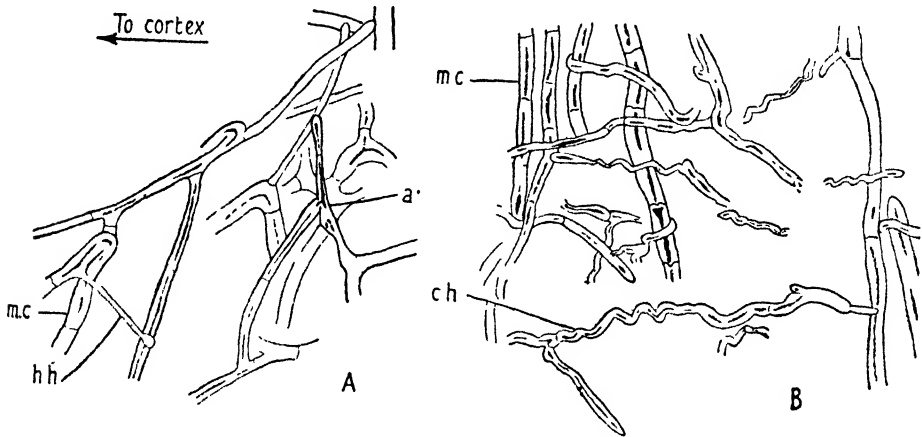
An interesting feature of the horizontal growth of these hyphae is that many of them follow a zigzag course or are coiled (Text-fig. 3 B, and Pl. VII c). This may be the result of some resistance met by the hyphae to their horizontal course, and it may serve to give the lamina greater resilience, enabling it better to withstand the compressions from the buffeting of the waves.

The formation of these horizontal hyphae is accompanied by the separation of some of them to form the air chambers, whilst others become more closely interwoven into the diaphragms separating the air chambers.

The formation of these horizontal hyphae suggests that the production of

the air chambers is an *active* process, and not merely the degeneration of the internal tissues, as suggested by Herriott (1923), and that it is more than a splitting caused by uneven rates of growth at the two surfaces, as is suggested by Grabendörfer (1885). The zigzag course of the hyphae indicates that it is the active growth of the hyphae which causes the splitting apart of the tissues, and that it is not caused by an internal accumulation of gases.

The air chambers usually extend from one surface of the lamina to the other with no central diaphragm. They vary considerably in size, but are



TEXT-FIG. 3. A, Cells from a transverse section about 5 cm. behind the apex where the air spaces are just forming. *m.c.*, medullary cells pulled out of their vertical course; *h.h.*, small horizontal hyphae; *a*, point of fusion of a hypha with another hypha or with a medullary cell. (Scale = $\times 250$.) B, Longitudinal section about 5 cm. behind the apex, where the air spaces are just forming, showing the spiral coiling of some of the hyphae. (Scale = $\times 250$.)

usually polygonal in shape, giving the centre of the lamina a honeycomb-like appearance (Text-fig. 1 A). The air chambers must give the lamina greater buoyancy and possibly serve as a reservoir for gases.

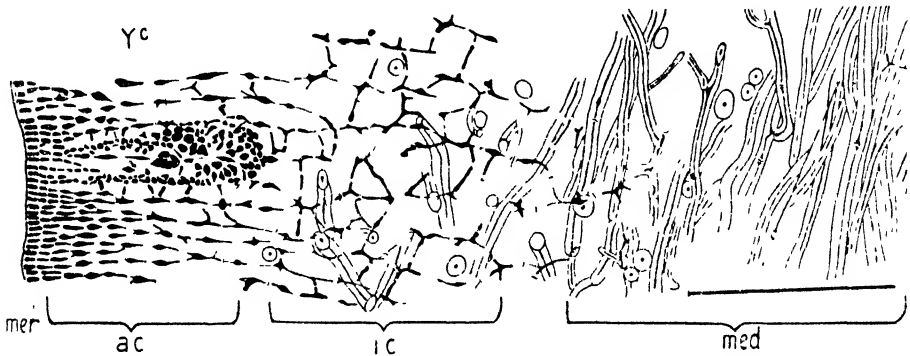
The fully developed lamina

The formation of the air spaces is accompanied by an increase in the thickness of the outer layers of the thallus and by a great increase in the number of hyphae, and within a few centimetres of the apex the mature structure is built up.

This consists (Text-fig. 4) of a *meristoderm*, inside which is an *outer cortex* about 10 cells deep and increasing in depth as the distance from the apex increases. These cells all have granular contents and are somewhat elongated in the direction perpendicular to the surface, and, being arranged in regular, radial rows, their derivation by tangential divisions from the meristoderm is obvious. The radial walls of these cells are very swollen, whilst the tangential walls remain sharply defined and highly refractive, thus emphasizing the arrangement in rows. Between the inner cells of this region are a number of large hyphae, running mainly in the tangential direction.

The outer cortex passes gradually into an *inner cortex*, the cells of which are drawn out into irregular shapes. This is probably due to the protoplasts having remained in contact with the tangential walls and the membranes of the pit connexions when the swelling of the radial walls occurred. Between the irregular reticulum formed by the protoplasts interweave numerous hyphae, running principally in the longitudinal direction.

Internally to this is a region much wider than either of these two outer regions, consisting almost entirely of longitudinally running hyphae, between which can be distinguished filaments of medullary cells and a few horizontal



TEXT-FIG. 4. Longitudinal section of the mature lamina, outer region only, in the plane perpendicular to the plane of flattening. *yc*, probable young conceptacle. Other letters as in Text-fig. 1. Before the central air chambers are reached, there is a further zone of hyphae, to the depth of a distance three times the length of the line marked at the side of the hyphae (Scale — $\times 125$.)

filaments of cortical cells. This region is separated from the central air chambers only by a very narrow zone of horizontal hyphae, from which grow out the small hyphae which form the diaphragms.

Herriott (1923) describes the lamina as consisting of two regions—cortex and medulla—similar to those here described, the cortex consisting of flask-shaped cells in radial rows perpendicular to the surface, and the medulla of 'filaments' parallel to the longitudinal axis bound together by transversely running filaments. Possibly the longitudinal 'filaments' are the hyphae and the horizontal one the innermost members of the radial rows of cortical cells.

Grabendorfer (1885) describes a central tissue of long, cylindrical cells and star-shaped cells—the latter probably corresponding to the inner cortical cells which are irregularly shaped. The former, which he describes as being united end to end in filaments ending blindly, are probably hyphae. Grabendorfer's figures show many points of similarity with the corresponding regions of my material (cf. Grabendorfer, Figs. 4 and 5 with Text-figs. 1 c and 4).

Growth of the lamina

Although there is no specialized initial cell, there is localization of active growth in length to the tips of the lamina segments. This is indicated both

externally by their lighter colour and internally by the progressive differentiation of the tissues with increasing distance from the apex. This active growth is brought about by the horizontal, anticlinal divisions of the meristoderm in this region, and is also accompanied by a general increase in size all over, also brought about by the divisions of the meristoderm.

Branching seems in many cases to be initiated by small, lateral outgrowths arising shortly behind the apex. These are characterized by the extra meristematic activity of the meristoderm and are accompanied by the up-growth of hyphae from the parent segment into the young branch.

The splits of the lamina

The lamina of *D. antarctica* is perforated by a number of splits, varying in size from a few millimetres to about 2 in. These splits are surrounded by a cortex continuous with that of the exterior and so complete that no trace of any rupture or disorganization of the tissues can be seen.

The cortex surrounding the split seems to be organized *before* the split is formed. Before the split actually breaks through, its future position is visible externally as a brown line. In this stage considerable internal activity can already be seen. The inner cells become arranged into regular rows like those of the outer cortical cells, but extending deep into the tissues of the lamina and arranged so that they lie perpendicular to the line of the future split (Pl. VII E).

When the cells have become arranged in this manner, the line of the split becomes apparent as a dark brown line, perpendicular to the surface, in the middle of the region of activity (Pl. VII F), and is apparently due to the degeneration of the cells in this region.

Finally the split breaks through, and when this happens a complete cortex is already formed, so that the underlying cells are never exposed by the rupture (Pl. VII G). The process of the organization of the cortex and the final rupture take place from the outside to the centre, so that when the split first begins to break through, the entire process is complete in the outer regions, whilst the centre may still be in the stage of the organization of the cortex. Similar stages in development may be seen both in the regions which have not broken through at all and also in the regions just beyond the limits of the already-existing splits.

These splits in the lamina of *D. antarctica* are not at all a common feature of the Fucales, but are much more characteristic of the Laminariales, where the occurrence of such splits is quite a regular feature, and in some species it is the usual method of subdivision of the frond into smaller segments. Amongst the Laminariales there seems to be no consistency in the manner of split formation, different methods being recorded for different species (Humphrey, 1886; Macmillan, 1899). Usually the meristoderm is responsible for the closure of the split, but it generally extends around the exposed surface after the split has formed. In the Fucales irregular splitting and tearing of the lamina is not at all uncommon, when a rather irregular tissue is organized to

close the wound, but there is not the production of a regularly arranged cortex as in *D. antarctica*.

The function of these highly organized splits in *D. antarctica* is not clear. It is not possible to say from the examination of a single specimen whether they assist in the subdivision of the frond or whether, by allowing the water to pass through them, they merely serve to lessen the resistance of the lamina as it swirls through the water.

The solid, basal 'palm' of the lamina

The structure of the solid, basal palm of the lamina differs very little from that of the basal portions of the inflated portion of the lamina, except that here, instead of the central region being occupied by central air chambers, it is filled with interweaving hyphae. These central hyphae have a lumen of slightly greater diameter than the outer ones and have thinner and less deeply staining walls.

The outer cortex is deeper here than in the inflated portion of the lamina, the depth increasing with the distance from the apex, until at the base of the palm it is about 20 cells in depth. Of these, the outermost have dense, granular contents and are only slightly elongated radially, and with the increasing thickness as the palm passes gradually into the stipe this radial elongation becomes more pronounced. The tendency towards the vertical elongation of the inner cortical cells seen higher up in the lamina is no longer apparent, any tendency towards elongation now being in the horizontal direction.

These three main tendencies seen in the palm—the formation of a central tissue of swollen cells, the greatly increased depth of the cortex, and the radial elongation of the inner cortical cells—foreshadow the condition in the stipe, where they are all developed to an even greater extent.

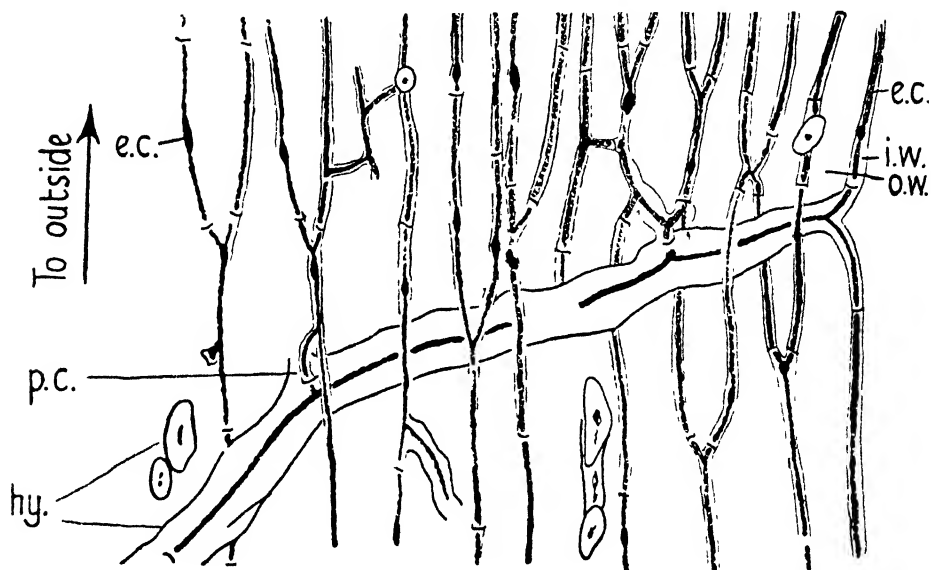
The stipe

At its lower edge the palm passes gradually into the stipe, which has basically the same structure, but shows radial instead of bilateral symmetry.

The outer cortex is much thicker than in the lamina, being from 20 to 40 cells in depth but still showing the arrangement of the cells in radial rows perpendicular to the surface. The outer 10 to 20 cells—the number increasing towards the base of the stipe—are slightly elongated in the direction perpendicular to the surface and possess very dense contents. They probably constitute the assimilatory tissue described by Herriott (1923).

The cells of the meristoderm are slightly larger than those in the lamina and are broader in comparison with their width, a feature probably correlated with the fewer anticlinal divisions in this region. Often the cells are broader at their outer ends than at their inner ones (Text-fig. 2 D). Fritsch (1945) describes the meristoderm cells of *Halidrys* as being larger towards the base of the axis than near the apex.

Farther from the exterior the cells of the cortex are radially elongated to about 10 times their width (Text-fig. 5). As in the lamina, their radial walls are very swollen so that their protoplasts are widely separated. Here, as in the medulla of the lamina, a layer of the wall immediately surrounding the protoplast stains red with Gentian violet, the reaction being more pronounced towards the centre of the stipe. The remainder of the swollen region of the wall did not stain at all with Gentian violet, and, in tangential longitudinal



TEXT-FIG. 5. Transverse section of the stipe through the elongated cells (*e.c.*) of the cortex, showing the origin of a hypha from a cortical cell, and secondary pit connexions (*p.c.*) between it and other cortical cells. *i.w.*, inner, staining portion of the wall, *o.w.*, outer, non-staining portion of the walls. (Scale = $\times 400$.)

sections, a fine, deeply staining membrane could be distinguished in the centre of this non-staining region (Text-fig. 6 A).

The very great radial elongation of the cortical cells may be correlated with the principal direction of growth, which, in this region, is in the horizontal direction, contrasting with the increase in length in the lamina. This is also seen in the divisions of the meristoderm, since in the stipe there are very many periclinal divisions and few anticlinal ones (Text-fig. 2 D). The preponderance of periclinal divisions over anticlinal ones is also noted by Fritsch (1945) in the basal regions of *Halidrys* and is also seen in the basal rounded portions of the axis of *Scytothalia dorycarpa* (Naylor, in press).

A few tangentially running hyphae interweave between the radially elongated cortical cells, from which some of them can be seen to originate (Text-fig. 5 and Pl. VII D). Some also have pit connexions with the cortical cells. These hyphae are of greater diameter than those of the lamina, and slightly less deeply staining.

Towards the inner region of the radially elongated cortical cells numerous hyphae interweave between the cortical cells which become less numerous until a zone is reached consisting almost entirely of longitudinal hyphae, between which can be detected the innermost members of the cortical cells, either as horizontal filaments or as a rather irregular reticulum similar to that at the inner edge of the cortex in the lamina. These hyphae probably form the 'filamentous conducting tissue' described by Herriott, and the innermost cortical cells between them are probably her 'transversely running filaments of cells serving to bind together the longitudinal filaments and make a firmer tissue'.

This zone of hyphae is fairly narrow—occupying about one-twentieth of the total diameter of the stipe—the hyphae gradually becoming more swollen towards the interior of the region until the central region is reached. This occupies about four-fifths of the total diameter of the stipe, and consists of longitudinally running filaments of extremely swollen cells (Text-fig. 6B), the degree of swelling increasing towards the centre, the lamina of the cells at the centre having about 10 times the diameter of those at the outside. A few filaments whose cells are not so swollen occur at intervals between the very swollen ones. It was not possible to determine whether these very swollen filaments were hyphal or medullary in origin.

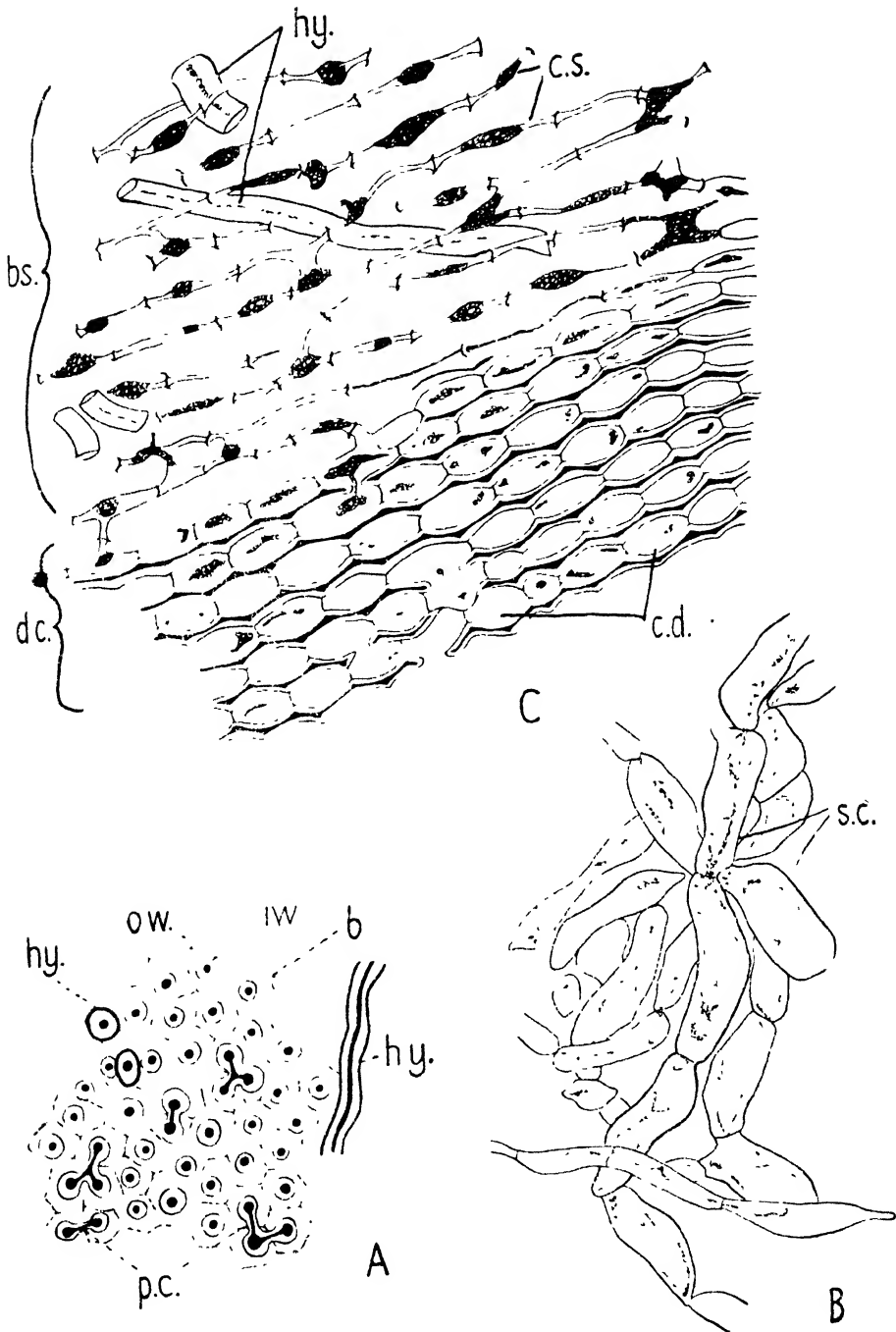
Around the centre of the stipe is a ring of cells where the swollen cells give rise to hyphal-like outgrowths with very dense contents, which penetrate through and between the cells of this region. The dense contents of the cells make this region appear to the naked eye as a black cylinder.

The cracks of the stipe and palm

The cracks in the stipe are about 1–2 mm. deep, extending deep into the cortex. Like the splits in the lamina, the cracks are bounded on both sides by radial rows of cortical cells arranged in rows perpendicular to the surfaces (Pl. VII B).

Such an arrangement might well be produced by differential activity of the meristoderm, which would throw the surface into a series of folds. This, however, does not appear to be the case, as places were found where the cells of the cortex showed an arrangement as if around a crack, yet no signs of any split were yet visible, the region of the future crack being occupied by cortical cells (Pl. VII A). Thus it seems that the cortical cells are stimulated to activity, probably as Herriott suggests, by stresses and strains due to the weight of the lamina, and the split appears later, probably after the cortex has been organized. I do not think that the lack of any split is due to the closure of a previously formed split, since the cell arrangement is so regular on both sides of the line of demarcation; there is no sign of any disorganization at the base of the crack such as might be expected if it had once been open; nor is there any sign of abundant mucilage cementing the crack together. Moreover, the hyphae run across the area without any interruption (Pl. VII A).

The idea that the cracks form in relation to stresses and strains due to the weight of the lamina is further supported by the presence of secondary cracks



TEXT-FIG. 6. A, Tangential *longitudinal* section through the elongated cortical cells of the stipe. *b* = Outermost layer of wall. Other letters as in Text-fig. 5. (Scale = $\times 400$) B, Radial longitudinal section of the medulla of the stipe, showing a longitudinal filament of very swollen cells. (Scale = $\times 180$.) (Cf. medullary cells of the lamina in Text-fig. 1 d, which is drawn to a scale more than twice as great as this.) C, Radial longitudinal section of the cortex at the extreme base of the stipe (*b.s.*), showing the change to disc structure. *c.d.*, cortical cells of the disc; *c.s.*, cortical cells of the stipe; *d.c.*, disc. (Scale = $\times 400$.)

within the main cracks, and these only on their upper surfaces. These smaller cracks repeat the structure of the larger ones with the cortex continuous around their edges, and thus give an even greater range of bending.

The cracks on the basal portion of the palm are not nearly so deep and appear to be merely splits in the outer tissues, and show no organization of a cortex around the torn edges.

Transition from stipe to disc

Towards the base of the stipe there is an increase in the number of outer cortical cells with dense contents, accompanied by a lesser degree of elongation of the inner ones and by a decrease in the extent of the outer zone of unswollen hyphae. The region of the stipe just above the disc thus possesses a wide cortex, the inner cells of which are fairly elongated, and between which numerous hyphae interweave. There is no region of gradual transition from this structure to that seen in the disc, but, within a few rows of cells, the cortical cells change from elongated cells with swollen walls and narrow protoplasts to brick-shaped cells with firm unswollen walls with deeply staining middle lamellae and no interweaving hyphae (Text-fig. 6 c).

The boundary runs obliquely across the rows of cells, so that, in some rows, whilst the outer cells have the form of disc cells, the inner ones may still retain the structure of the inner cortical cells of the stipe (Text-fig. 6 c). This obliquity of the boundary probably gives greater strength in this region than would result if the change proceeded at an equal rate in all the rows of cells, as the straight boundary which would result would probably lead to a tendency to splitting.

The disc

A median vertical section of the entire disc shows it to consist of a central, pale, and rather soft portion, surrounded by an outer dark portion, very hard and woody to cut. The central pale region is continuous with the central region of the stipe and is likewise composed of large, swollen cells.

The outer dark portion consists of cells arranged in regular rows radiating from the central pale portion to the upper surface of the disc (Text-fig. 7). The cells are roughly cubical in shape and have a large lumen containing a few granules. The anticlinal walls of these cells are strongly thickened, but the periclinal walls are very thin. There are frequent thin-walled regions in the thick anticlinal walls, and in places these walls appear to have broken down altogether, leaving complete continuity between the cells (Text-fig. 6 c). The middle lamellæ stains deeply with Gentian violet, but the rest of the wall does not stain at all.

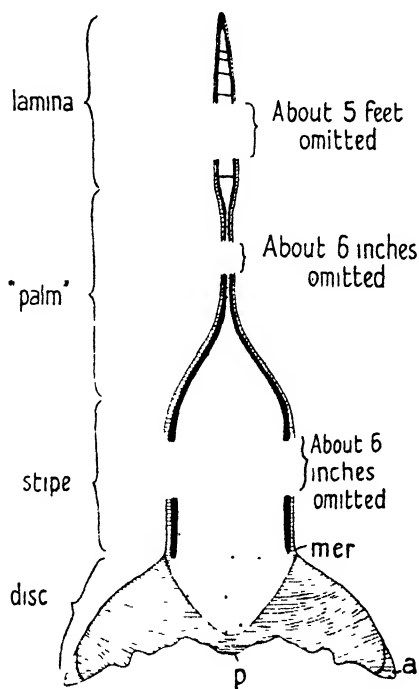
The upper surface of the disc is bounded by a meristoderm and an outer cortex about 6–10 cells in depth. These cells are small and cubical with thin walls and very dense, granular contents. The complete continuity in arrangement between these cells and the larger thick-walled cells suggests that the

latter are also cortical cells derived from the meristoderm by periclinal divisions. Thus the whole of the expanded portion of the disc consists of cortical cells.

The arrangement of the cells in regular rows is not obvious in all parts of the disc, particularly towards the lower surface, where there are many interruptions due to the irregularities of the rock surface and to the burrowings of marine molluscs whose shells are in places embedded in the disc. The cells at the lower surface of the disc in contact with the rock are irregular in shape and have brown contents.

At the outer margin of the disc a further zone of cells could be distinguished between the outer thin-walled assimilatory cells and the inner thick-walled cells. This layer, about 10 cells in depth, consists of irregularly shaped cells with fairly thick walls and dense granular contents. As the disc increases in size from year to year, possibly this region is in process of transition from assimilatory tissue to the permanent tissue of the centre of the disc (Text-fig. 7 at *a*).

This description of the disc does not compare very well with Herriott's descriptions, either of a very young disc or of an older one which is described as consisting of 'irregularly shaped cells embedded in a woody matrix'. An irregular appearance is seen, however, if the sections run obliquely through the regular rows of cells. Grabendörfer's description more closely resembles mine as he describes the disc of *D. Harveyi* as consisting of cubical or prismatic cells arranged perpendicular to the upper surface



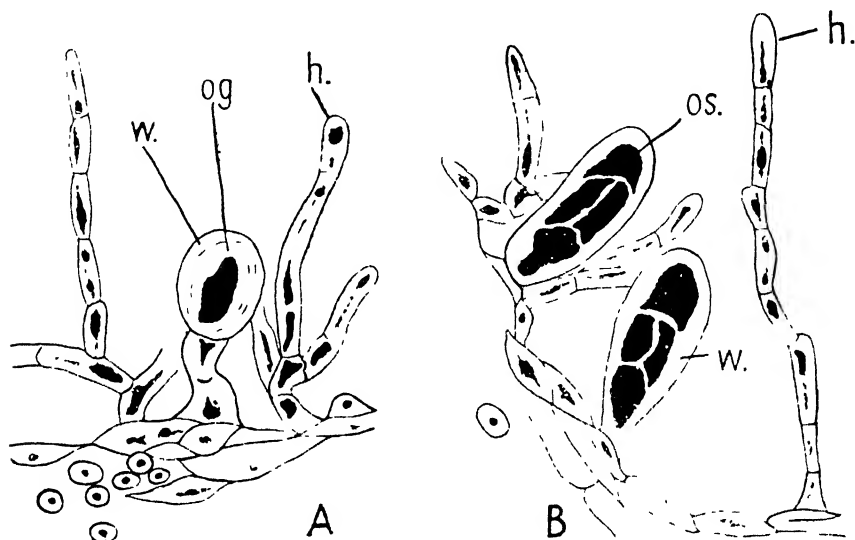
TEXT-FIG. 7. A diagrammatic longitudinal section of the whole plant in the plane perpendicular to the plane of flattening, to show the distribution of the tissues. *mer*, meristoderm; *a*, actively growing margin of the disc; *p*, possible point of attachment of the young plant. The direction of the lines of shading in the outer cortex indicates the direction of the files of cells derived from the meristoderm. (Scale = $\frac{1}{2}$ natural size in the horizontal direction. Not to scale in the longitudinal direction.)

That the broad, expanded portion of the disc consists of cortical cells is indicated both by their continuity through the outer cortex, with the meristoderm, and by their continuity with the cortical cells of the stipe (Text-fig. 6 c). Thus the disc differs from that of *Fucus*, which is formed by the segmentation of outgrowing hyphae, but more closely resembles the primary disc of the Laminariales, which is formed by the active tangential divisions of the meristoderm (Wille, 1897; Killian, 1911).

The conceptacles

D. antarctica is dioecious (Herriott, 1923), and the plant under investigation is a female one bearing oogonia in conceptacles scattered all over the lamina.

The conceptacles on the palm were almost spherical in shape, opening to the exterior by a rather irregular ostiole. These were old conceptacles, as they contained numerous empty exochitons and some mature oogonia. In addition there were a few young oogonia, so probably there is a succession of ripe



TEXT-FIG. 8. Oogonia and sterile hairs from the walls of the conceptacles. A, Oogonium showing stratification in the swollen wall. B, Oogonium showing differentiation into four oospheres. *og.*, oogonium, *os.*, oosphere; *w.*, wall of oogonium, *p.*, paraphysis. (Scale = $\times 400$.)

oogonia within the conceptacle. These oogonia and empty exochitons were borne directly on the wall of the conceptacle—no basal cell was seen—and were interspersed with moniliform hairs.

In these conceptacles there were only a few of the branched oogonial hairs described by Herriott.

The conceptacles on the inflated portion of the lamina, on the other hand, contained numerous, much-branched hairs, some of which bore oogonia. There were also many oogonia borne directly on the walls.

The conceptacles of the inflated portion of the lamina were slightly larger than those of the solid palm, and were elongated in the direction of the longitudinal axis—a feature possibly correlated with the growth in length of the lamina. They were younger than those of the palm, containing only a few mature oogonia and many young ones.

The oogonia showed two principal phases of development. In some cases they were almost spherical with very swollen walls in which numerous layers could be seen (Text-fig. 8 A). Others had an elongated protoplast divided into

four portions and had much less swollen walls (Text-fig. 8 B). These four portions of the protoplast are probably the four oospheres, since previous workers (Skottsberg, 1907; Herriott, 1923) have described the oogonium as containing four oospheres. If the spherical oogonia with the undivided protoplast are the young oogonia, then the condition is unusual amongst the Fucales, since the oogonial wall is usually unswollen in the young stages, becoming swollen as the oogonium approaches maturity, when it plays a part in the liberation of the contents.

No ostiole could be distinguished in the conceptacles on the inflated region of the lamina, either by a surface examination with a binocular microscope or by series of microtome sections. In the region where the ostiole might be expected to occur, the cells of the cortex are arranged as if around a split, although no split is visible (cf. the splits of the stipe and lamina). This arrangement of the cells, together with the irregular nature of the ostioles of the conceptacles on the palm, suggests that the ostioles may be formed at a later stage by the separation of the cells. Grabendörfer (1885) was also unable to find any opening to the exterior in the young conceptacles of the material of *D. Harveyi* that he examined. Tahara (1913) describes the conceptacles of *Cystophyllum sisymbrioides* as lacking an ostiole, and the liberation of the oospheres is here preceded by the growth of the paraphyses through the outer wall of the conceptacle. This condition differs from that in *Sargassum Horneri* (Tahara, 1913) and *Bifurcaria tuberculata* (Rees, 1933), where the ostiole is closed by a 'plug' formed from the paraphyses.

As well as the hairs and the oogonia, the conceptacles of *D. antarctica* contain numerous hyphae which grow through the wall from the cortex. Such hyphae are seen, but to a lesser extent, in the conceptacles of the palm. This feature is also described by Grabendörfer for the older conceptacles of *D. Harveyi*, but is not recorded elsewhere in the Fucales.

Skottsberg (1907) describes the shedding of the four oospheres from the exochiton and their escape from the mesochiton, in a manner similar to that occurring in *Fucus* (Thuret et Bornet 1878), but the release of the spermatozoids and fertilization have not been described in *Durvillea*.

The distribution of cellulose

Iodine and concentrated sulphuric acid were used to test for the presence of cellulose.

In the young tips cellulose is present in the walls of the hyphae only, where the reaction is very pronounced. A few centimetres behind the apex traces of cellulose can be detected in the walls of the medullary cells in a very thin layer of the wall immediately surrounding the protoplast.

In the older portions of the lamina cellulose is present in the walls of the horizontal hyphae forming the diaphragms, in the walls of the longitudinal hyphae, and in the walls of the medullary cells. In the hyphae the cellulose is present throughout the walls, but in the medullary cells it is confined to the region immediately surrounding the protoplast. It could not be detected with

any certainty either in the walls of the cortical cells or in the meristoderm, but Grabendörfer (1885) records the presence of cellulose in both the cortex and the meristoderm, similarly restricted to the layer bounding the protoplast. He also describes it in the cylindrical cells of the medulla (= hyphae?), where it is present throughout the walls.

There is no cellulose in the lining layer of the conceptacles nor in the oogonia or the monoliform hairs, but it is present in the walls of the hyphae which penetrate through the conceptacles.

In the central region of the stipe the walls of the very swollen cells give a pronounced cellulose reaction, which is restricted to the region of the wall next to the protoplast. In the outer region of the stipe there is a cellulose reaction in the walls of the hyphae, and traces are seen in the innermost cortical cells.

In the disc cellulose is present in all the walls except those of the assimilatory cells. In the central region the reaction is the same as in the centre of the stipe. In the expanded portion the bulk of the firm thickened wall remains colourless, but a very thin layer around the protoplast stains blue.

Thus, cellulose appears to be present throughout the walls of the hyphae, wherever they occur in the plant. In the cells of the medulla and the cortex, on the other hand, it is restricted to the part of the wall lining the protoplast. There seems to be a progressive differentiation of cellulose from the central regions to the outside with increasing distance from the apex. In the lamina it is present in the walls of the medulla only; in the stipe it extends to the inner cortical cells; whilst in the disc it is present in all the cells except in the outermost (assimilatory) cells. The restriction of the cellulose to the lining layer of the walls of the cortical cells and its presence throughout the walls of the hyphae is also described by Naylor and Russell-Wells (1934) for *Fucus vesiculosus*, but in addition they found it in concentric bands in the thicker-walled medullary cells, a condition not seen in *D. antarctica*.

The consistent occurrence of cellulose throughout the walls of the hyphae and its limitation in the disc to the lining layer of the walls is, I think, a further indication that the disc is not formed from hyphae but from cortical cells.

Comparisons with the Fucales and the Laminariales

The elongated, strap-like thallus of *D. antarctica* bears a superficial resemblance to the form of the thallus of many of the Laminariales. Both Oltmanns (1889) and Grüber (1896) compared it with *Ecklonia*. Hooker (1847) compared it with *Himanthalia* on account of the much divided fronds bearing conceptacles all over their surfaces. *D. antarctica* is a perennial, and the lamina increases in size from year to year, and is not characterized by the annual blade-renewal seen so frequently amongst the Laminariales.

The attaching organ of *L. antarctica* is a roughly circular disc, externally like those of the Fucales. Internally, however, the disc differs from those of the Fucales, as it appears to be built up by the active tangential divisions

of the meristoderm, as is the primary disc of the Laminariales, rather than by the segmentation of down-growing hyphae, as in the Fucales.

Although differing from the Fucaceae and the Sargassaceae in the lack of any apical groove containing a special initial cell, the lamina of *D. antarctica* possesses a certain amount of apical growth, as can be seen externally by the lighter colour of the tips, and internally by the study of the differentiation of the tissues behind the apex. Growth seems to be entirely carried on by the activity of the surface layer of cells. The nearest approach to this condition in the Fucales is that in the Hormosiraceae, where growth is carried on by a small group of initials. In the possession of apical growth *D. antarctica* more closely resembles the Fucales than the Laminariales, where growth is intercalary.

Organized splits in the thallus, such as seen in *D. antarctica*, are not a feature of the Fucales, but are of common occurrence amongst the Laminariales, where they often constitute the normal method of subdivision of the frond. In the possession of similar splits *D. antarctica* more closely resembles the Laminariales than the Fucales, but it is doubtful whether in *D. antarctica* they form the method of subdivision of the frond.

The internal organization of *D. antarctica* resembles that of the Fucales and consists, in the young part of the lamina, of a meristoderm, cortex, and medulla, very similar to the corresponding tissues of *Fucus*, although the component cells are very much smaller. These three tissues can be distinguished throughout the plant, although in the older parts of the lamina the arrangement is obscured by the great production of hyphae, and in the stipe by the swollen nature of the cells. The meristematic activity of the meristoderm is very marked at all levels of the plant—rather more so than is usual in *Fucus*. The fusion of the hyphae with other cells resembles the condition in the Laminariales rather than the Fucales.

In its method of reproduction, so far as known, the affinities of *D. antarctica* are definitely with the Fucales. The Oogonia are borne in conceptacles and contain four oospheres. In the material available the cytology of their development could not be worked out.

Although externally the thallus of *D. antarctica* resembles that of a laminarian, in its internal organization and in its method of reproduction, its affinities are definitely fucalean. On the whole, *D. antarctica* is probably best regarded as a member of the Fucales.

The early stages of development of *D. antarctica* do not seem to be known, and a study of them might help to throw further light on the affinities of this genus.

SUMMARY

The plant under investigation is a young plant of *Durvillea antarctica*, a giant seaweed of antarctic and subantarctic waters. The thallus consists of an attaching disc, a cylindrical stipe, and a much-divided lamina composed of numerous strap-like segments. The form of the thallus closely resembles that of some of the Laminariales.

The thallus possesses a certain amount of apical growth, as is indicated externally by the lighter colour of the tips, and internally by their lack of differentiation, and by the gradual differentiation of the tissues behind the apex. There is no apical groove nor specialized apical cell, as is usual amongst the Fucales, but the apex is dome-shaped, bounded by a very actively dividing surface layer. This apical growth is supplemented by a general increase in size due to the activity of the entire surface layer.

The plant is composed of three principal tissues—meristoderm, cortex, and medulla—similar to those of *Fucus*. Numerous longitudinally running hyphae, and some horizontal ones, are produced as outgrowths from the cortical and medullary cells.

The mature lamina is inflated by a honeycomb-like central system of air chambers. These are initiated near the apex by the formation of spirally coiled, horizontally growing hyphae. The close interweaving of these hyphae is responsible for the formation of the diaphragms separating the air chambers.

The periclinal divisions of the meristoderm increase with increasing distance from the apex and are responsible for the increase in thickness of the stipe, and possibly also for the production of the disc.

There are a number of transverse cracks around the stipe and longitudinal slits in the lamina. In both cases a cortex and meristoderm appear to be organized *before* the splitting apart of the tissues, so that the underlying tissues are never exposed.

The disc seems to be formed from cortical cells with specially strengthened walls.

Cellulose is present throughout the walls of the hyphae and in the walls of some of the medullary and cortical cells in the layer of the wall immediately surrounding the protoplast. The number of cells with cellulose in their walls increases with distance from the apex, appearing first in the cells of the medulla. The cells of the disc contain cellulose only in the layer of the wall surrounding the protoplast.

Female conceptacles, containing oogonia borne either directly on the walls or on branched hairs, are scattered all over the lamina. The oogonia showed two principal stages of development—either they were spherical, with swollen walls and an undivided protoplast, or they were slightly elongated, with thin walls and a protoplast divided into four portions. The hyphae frequently penetrate through the conceptacles.

Although externally, in the form of the thallus, *D. antarctica* resembles a laminarian, its anatomical construction and method of reproduction are definitely fucal.

Since the completion of this account of the mature plant of *D. antarctica*, I have received some young plants from Professor V. J. Chapman, and I hope to be able to give an account of these at a later date.

This investigation was carried out in the Botanical Department of Westfield College, and I wish to express my thanks to Dr. E. M. Delf for her advice

and encouragement during its course and for her help in the preparation of this paper for publication. I am also indebted to the Botanical Research Fund for a grant which has made it possible to carry out this investigation.

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DESCRIPTION OF PLATES

Illustrating M. Naylor's article 'Observations on the Anatomy of *Durvillea antarctica* (Chamisso) Hariot'.

PLATE VI

The young plant of *Durvillea antarctica* under investigation, photographed on its arrival from New Zealand. The line at the left-hand side represents a distance of 3 ft.

PLATE VII

A and B. *Stages in the formation of the cracks of the stipe.*

A, R.L.S. before the cracks had opened, showing the cortical cells arranged in rows perpendicular to the line of the future crack, and showing the hyphae running across the crack.

B, R.L.S. after the crack had formed, showing the cortex organized around it.

C, L.S. in the plane perpendicular to the plane of flattening about 5 cm. behind the apex where the air chambers are just beginning to form, showing the medullary cells and the spirally coiled horizontal hyphae. (Cf. Text-fig. 3 B.)

D, T.S. stipe, showing the radially elongated cortical cells with a hypha originating from one of them. (Cf. Text-fig. 5.)

E, F, and G. *Stages in the formation of the splits of the lamina.*

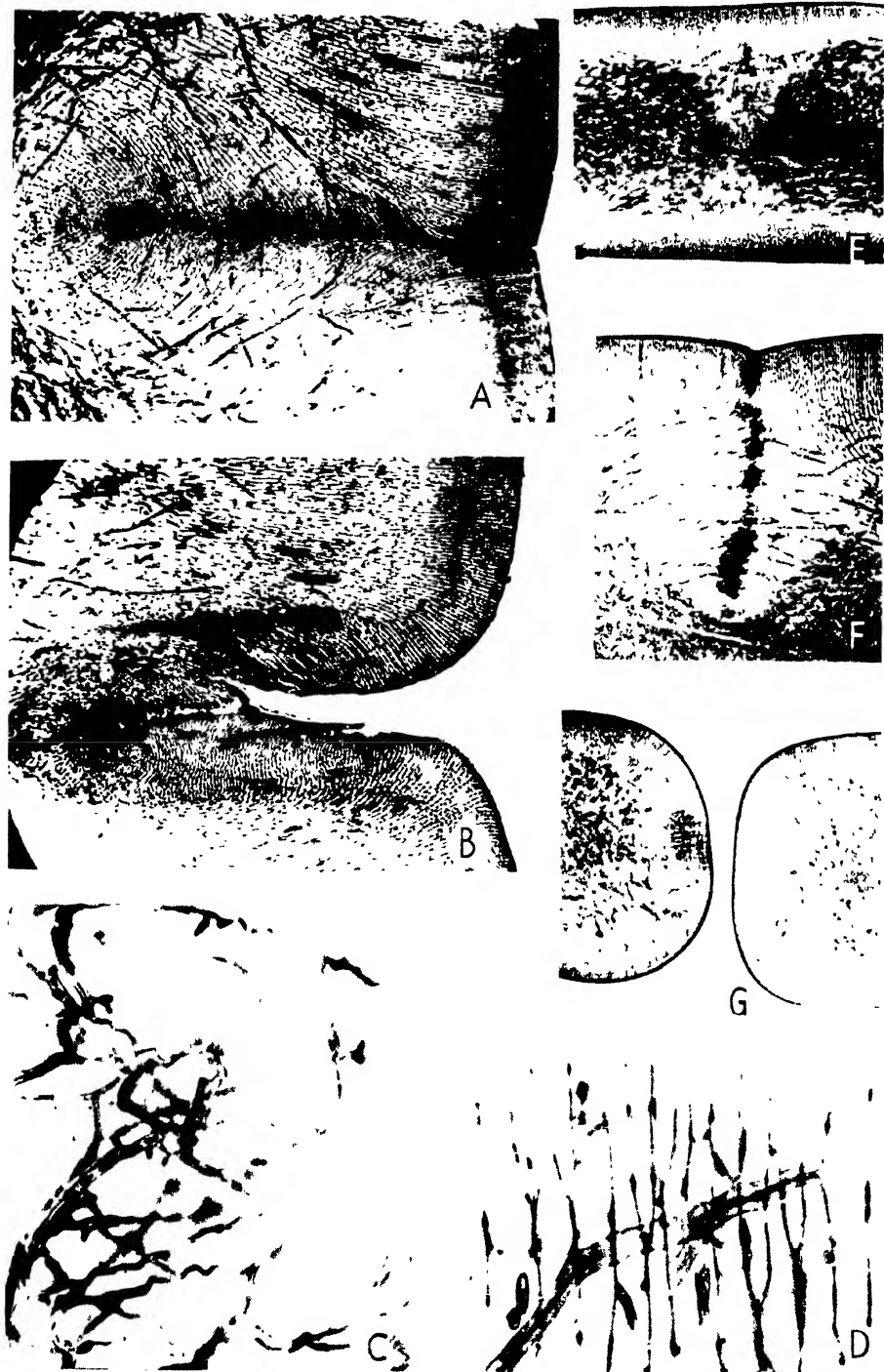
E, Very early stage. No external signs of the split are yet visible, but internally the rearrangement of the cortical cells can be seen.

F, A slightly later stage. Externally a brown line can be seen on the thallus, and internally this can be seen to be due to the degeneration of the cells in the region of the future crack.

G, The completely formed split with a continuous cortex around its inner margins.



M. NAYLOR. OBSERVATIONS ON THE
ANATOMY OF *DURVILLEA*



Studies in Stomatal Behaviour

III. The Sensitivity of Stomata to Mechanical Shock

BY

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With Plate VIII and nine Figures in the Text

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INTRODUCTION

IT has for long been generally accepted that the stomata of most plants are sensitive to mechanical shock, responding thereto by a temporary partial closure; and it is therefore reasonable to expect that the fixing of a porometer-cup will itself constitute sufficient shock to cause such closure. This possibility was first investigated by Knight (1916). He found that by the time a porometer-cup was in position the stomata were only slightly open; that the stomata then opened steadily in light, reaching maximum aperture after about 2 hours; and that subsequent observations on the same group of stomata showed that they would always return to this wide-open position on illumination after a period of darkness. He naturally concluded that this wide-open position represented the aperture normally attained by the stomata in light, but that the fixing of the porometer-cup caused a partial closure so rapid that the movement was completed by the time a reading could be taken.

The phenomenon was specifically investigated by Knight for *Eucharis mastersii*, but he mentions that it is also shown by *Ficus elastica*, and it appears

from his diagrams that the same is true of *Begonia*. It is not clear when it was first shown to be true also of *Pelargonium*, but it is evident from Heath's early paper (1938) on shock-response that the phenomenon was by then familiar.

Knight's interpretation of the phenomenon remained unchallenged until the publication of preliminary notes by Heath and Williams (1948) and Heath (1948). This paper presents a detailed account of investigations which led up to the new interpretation there put forward.

MATERIAL AND METHODS

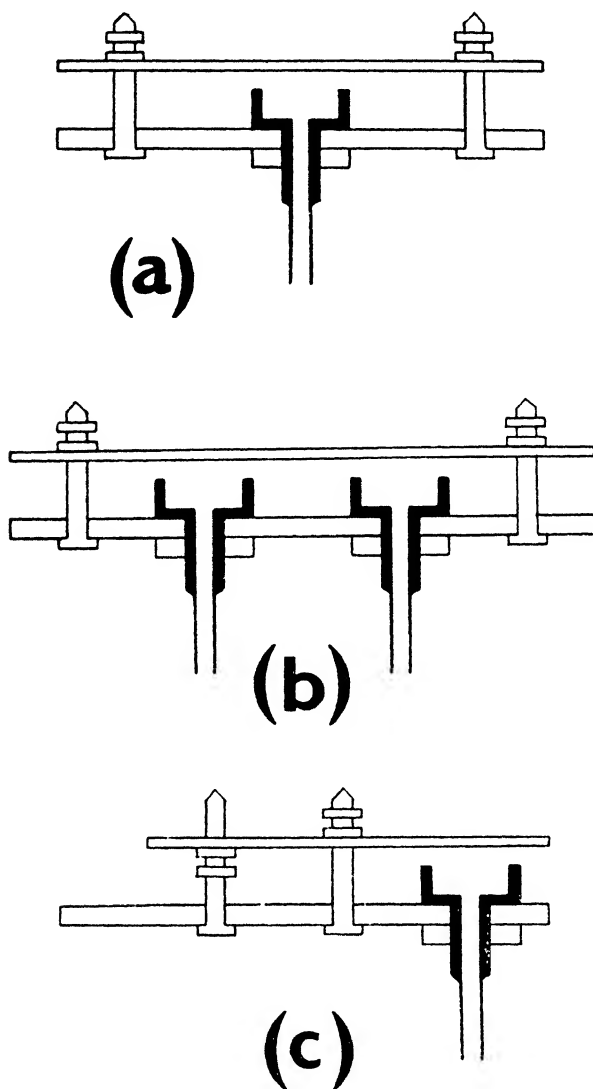
1. All experiments except No. 5 were carried out on *Pelargonium zonale* var. *Paul Crampel*; in the exception var. *Salmon Crampel* was used. The plants were propagated and grown in the Botany Garden of Bedford College, either in the greenhouse or in the open according to the time of year. The leaf under investigation remained attached to the plant in all cases.

2. The porometer experiments were carried out with a simple form of resistance porometer (Gregory and Pearse, 1934), essentially similar to that used by the author in previous observations on heat-shock phenomena (Williams, 1948a). A simple brass porometer-cup was used, with the now usual glycerol-gelatin washer and beeswax-vaseline luting-wax. The precise method of fixing is important since it has a direct bearing on the results obtained. The leaf was pressed down as gently as possible on to the washer, and a glass plate, located by means of two brass bolts on the porometer-cup holder, lightly dropped on to the top. The complete cup, holder, and glass plate is shown diagrammatically in Text-fig. 1(a); the nuts were screwed down only so far as was necessary to ensure that the glass plate was closely applied to the leaf and parallel to the base of the holder. In general it should be possible to obtain an airtight seal without tightening the nuts for this specific purpose; if, usually as a result of the accidental inclusion of a main vein, an airtight seal could not be obtained without tightening the nuts, the cup was removed and fixed in a new position on the leaf.

For certain experiments it was necessary to attach two cups to the same leaf. A double-cup holder, as used in earlier work and shown diagrammatically in Text-fig. 1(b), was tried and discarded, as it proved extremely difficult to ensure that both cups were attached sufficiently gently; in most such cases, therefore, two of the simple holders (Text-fig. 1(a)) were separately attached. In the few remaining cases difficulties of space arose in such an arrangement, and a simple 'cantilever' holder (Text-fig. 1(c)) was used for the second cup. The conditions for independence of two cups so attached to the same leaf have been investigated by Williams (1948b).

The manometer reading (i.e. the quantity P_2 of Gregory and Pearse) was used as a measure of stomatal aperture, decreasing pressure representing stomatal opening; all ordinates in the diagrams represent negative pressures in centimetres of water, with a possible maximum ordinate of $P_1 = 12$ cm.

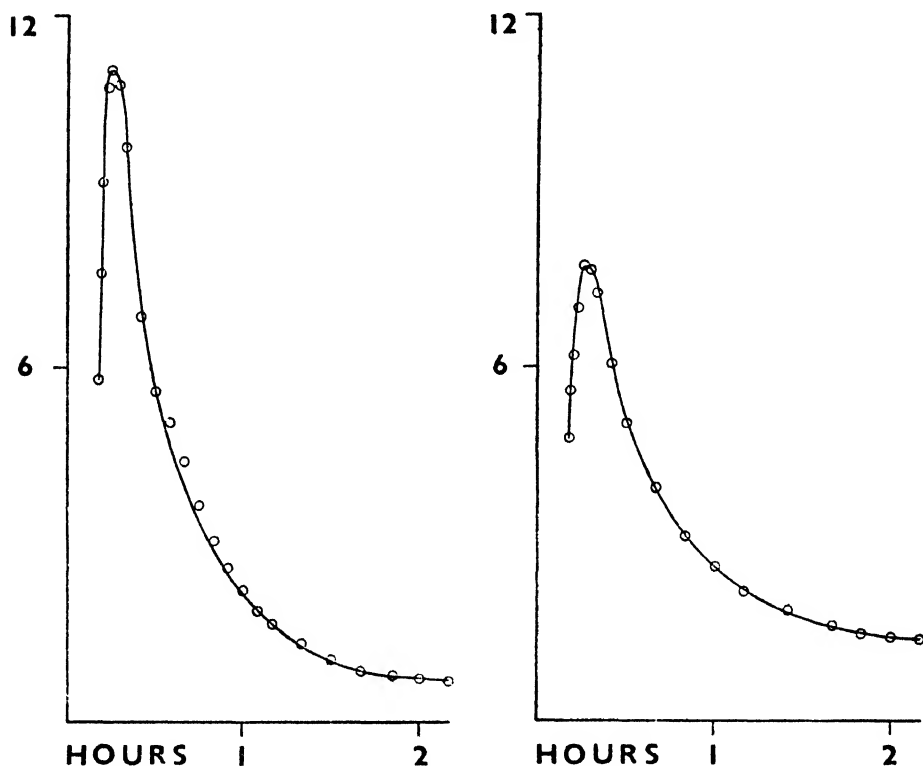
3. For other experiments a slight modification of the infiltration technique of Molisch (1912) was used. Wide-open stomata of *Pelargonium* are rapidly penetrated by absolute alcohol; stomata only slightly open are not. However,



TEXT-FIG. 1. Types of porometer-cup holder used in the investigation.

the resulting differences in appearance, though striking to the eye, are neither permanent nor easy to photograph. In the later experiments a solution of crystal violet in absolute alcohol was therefore used instead; no precautions were taken to ensure a standard strength, the solid dyestuff simply being added to alcohol (74 O.P. methylated spirit) to produce an intensely coloured, opaque solution. This was poured on to the leaf to be tested and allowed to

remain on the surface for 5 to 10 seconds (the time is not critical); the leaf was then held under a water-tap and the surplus dye solution washed off. Injected patches are by this means stained a vivid purple, and the leaf can be pressed in the normal way, providing a record whose permanence is limited only by that of the dye used. For photographic purposes a red dye would have been preferable, but none of the red dyes investigated was sufficiently intense.



TEXT-FIG. 2. Two examples of record obtained on first attachment of a porometer-cup in light, showing the Knight 'recovery' curve.

EXPERIMENTAL RESULTS

I. The Nature of the 'Shock-recovery' Phenomenon

1. The Knight effect

Experiment 1. Two examples of the typical record obtained when a porometer-cup is attached in light are shown in Text-fig. 2. It must be emphasized that the rapid rise at the beginning of each record does not necessarily imply stomatal movement; the manometers take an appreciable time to change position, and those in this apparatus took nearly 5 minutes to pass from the 'fully open' to the 'closed' position, though the greater part of this movement was complete within about 2 minutes. Although, therefore, this rapid rise could be due in part at least to stomatal closure, it cannot be taken as evidence

for such a movement; whether or no there is in fact a simultaneous stomatal movement will be considered later (vide § III, expt. 13).

Experiment 2. A few experiments were carried out to investigate whether there was any evidence of shock-transmission. A cup (A) was attached to a leaf and allowed to 'recover' in light until the porometer showed that the stomata were wide open (about 2 hours). A second cup (B) was then attached to the same leaf, and the manometer corresponding to cup A watched for signs of movement. No movement whatsoever of the stomata under cup A was discernible. The leaf under cup A is, of course, clamped more or less rigidly and should not itself be subjected to any direct mechanical disturbance when cup B is attached; the negative result therefore shows that, unlike the situation obtaining in heat-shock closure (Williams, 1948 *a*), no change of any kind is propagated across the leaf as a result of the shock. Examination of Knight's figures shows that this is true also of *Eucharis*, though he does not specifically mention the problem.

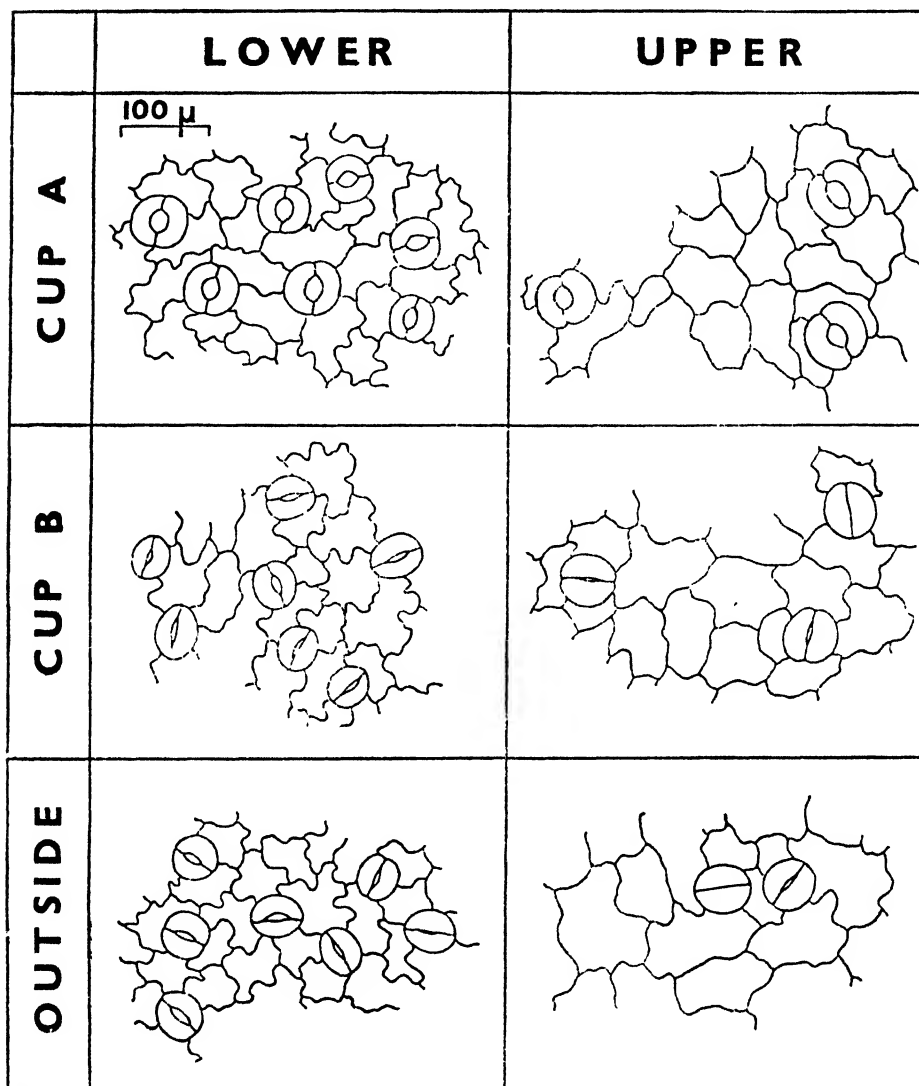
Experiment 3. It was considered desirable to investigate the state of affairs as revealed by Lloyd's (1908) stripping technique; for, as Knight pointed out, if the stomata of any given plant are so sensitive as to close when a porometer-cup is attached, the violence inseparable from Lloyd's method is likely to have the same effect. The experiment was carried out as follows: (1) a cup (A) was attached to a leaf and allowed to 'recover' in light for 2 hours as before; (2) a second cup (B) was attached to the same leaf; (3) when the manometer reading of cup B had reached its highest point, corresponding to maximum degree of closure and taking about 5 minutes from fixing, strips of epidermis were removed as quickly as possible from (i) within the cup B, (ii) within the cup A, and (iii) a portion of the leaf outside the cups, strips from both upper and lower epidermes being taken in both cases. The strips were plunged into absolute alcohol and examined later; camera-lucida drawings of portions of the strips are shown in Text-fig. 3.

Two points are noteworthy. First, there is no significant difference between the strip from under cup B and that from the leaf outside the cups, the stomata being almost closed in both cases; this is in keeping with the theory that the mere process of stripping has itself caused closure comparable with that resulting from the fixing of the cup. Second, the stomata on the strip from under the 'recovered' cup A are wide open—but *stripping has not in this case caused them to close*. Here, then, is the first intimation that all is not well with the simple theory put forward by Knight; if fixing the porometer-cup and stripping are each adequate to cause shock-closure, there should have been no difference between the stomata on the three strips. In fact, it would appear from this result that stomata, once recovered under a porometer-cup, are no longer sensitive to shock.

2. The effect of the glass plate

The first published intimation that Knight's theory was inadequate was a note by the author (Williams, 1947) drawing attention to the fact that the glass

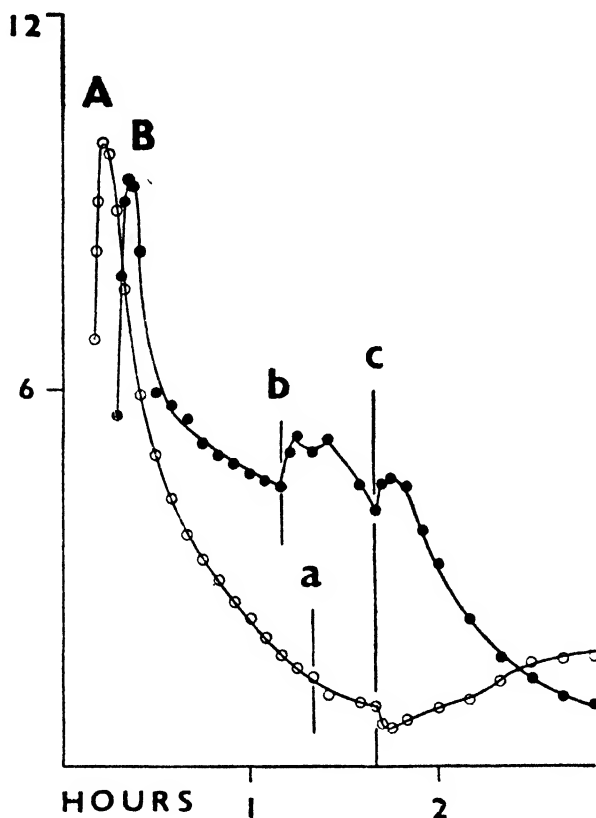
plate against which the leaf is pressed apparently plays an important part in the 'recovery' process. The experiment there described will be briefly recounted here, so that it may be seen in its proper relation to the rest of the investigation.



TEXT-FIG. 3. Camera-lucida drawings of portions of epidermal strips obtained in expt 3.

Experiment 4. A cup (A) was attached to a leaf in the normal way. A second cup (B) was attached to the same leaf but without the usual glass plate; this, though difficult, is possible if the consistency of the luting-wax is suitably chosen and great care taken in handling the leaf. The resulting records are shown in Text-fig. 4. Cup A (beginning of record to the point *a*) shows the normal 'recovery' curve; but the stomata under cup B (beginning of record

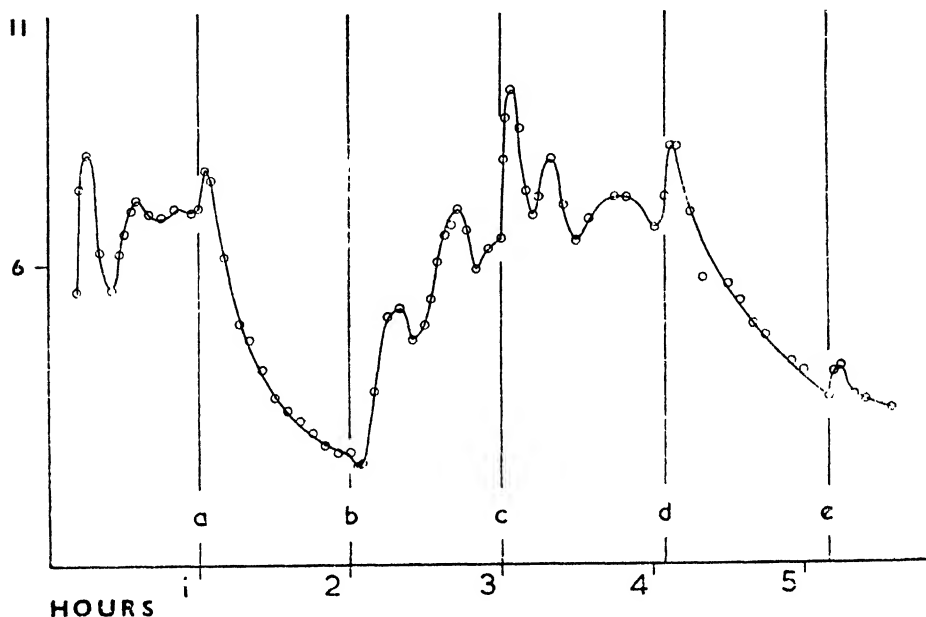
to the point *b*) which, there being no glass plate, might reasonably be thought to have suffered less mechanical disturbance, are opening more slowly and less regularly than those under cup A, and are, moreover, tending to a higher equilibrium resistance. The shock-sensitivity of the two sets was then tested by subjecting each for a few seconds to a sharp pressure from the forefinger



TEXT-FIG. 4. Effect of the presence of the glass plate on the 'recovery' curve, for explanation see text (expt. 4).

(at *a* on record A, at *b* on record B); in the case of A, the glass plate was removed for this purpose, then quickly replaced. The stomata under cup B are clearly shock-sensitive, responding with a rapid temporary closure; those under cup A are completely insensitive. To ensure that there were no inherent differences between the two sets of stomata the glass plate was removed from A and a similar plate clamped in the usual position on cup B (point *c* in Text-fig. 4). The immediate effect is a slight rise in apparent resistance at B and a similar fall at A, to be expected since the plate introduces a small additional resistance to air-flow. The further effects are striking: B for the first time promptly assumes the normal 'recovery' movement; and the stomata under A begin, slowly and irregularly, to close.

Experiment 5 shows the even more striking result obtained with the variety *Salmon Crampel*, the leaves of which, it is relevant to note, are appreciably less hairy than those of *Paul Crampel*. The record is given in Text-fig. 5. At the beginning of the experiment the cup was attached without a glass plate, and it will be observed that there was some irregular stomatal movement, but no progressive opening; the successive treatments to which the



TEXT-FIG. 5. Effect of the presence of the glass plate on the 'recovery' curve; for explanation see text (expt. 5).

cup was then subjected, represented by the lines *a*, *b*, & *c* on the record, were as follows:

- a*. Glass plate clamped in position.
- b*. Glass plate removed.
- c*. Mechanical shock (as in expt. 4).
- d*. Glass plate replaced.
- e*. Glass plate removed, cup subjected to mechanical shock, and plate immediately replaced.

This differs from the result of expt. 4 only in degree and in the slight shock-sensitivity shown at operation *e*; however, the stomata at this latter point were still opening quite rapidly, and it is likely that the very wide-open 'recovered' position had not yet been attained.

It is clear from these experiments that the presence of the glass plate either contributes to, or is responsible for, the normal 'recovery' phenomenon; and it is also clear that, as deduced from the observations on epidermal strips (expt. 3), the stomata, once wide open under a porometer-cup as normally

attached, are insensitive to mechanical shock. The writer first attributed this insensitivity to some form of 'accommodation' such as that shown by *Mimosa pudica*; however, since the glass plate does not press directly on the stomata under investigation, and since there is no succession of stimuli but only a steady pressure, this explanation seemed somewhat implausible.

3. Infiltration experiments

It was at this point in the investigation that Dr. O. V. S. Heath made the crucial suggestion that the phenomenon might not be one of recovery at all, but that the stomatal resistance recorded by the porometer at the moment of fixing might be a valid measure of the aperture normally attained on a leaf freely exposed to the air, the wide opening then observed being an abnormal manifestation occurring only under a porometer-cup. It was decided to test this hypothesis by the infiltration method described above.

Experiment 6. Two cups were attached and the leaf allowed to 'recover' in the normal way for 2 hours; the cups were then detached and the leaf removed from the plant and tested. The result is shown in Pl. VIII, 1; it will be seen that the areas under the cups are strongly injected, the rest of the leaf unchanged. This shows clearly that, at the moment of testing, only those stomata under the cups were wide open; but it could be argued that this was because those stomata were 'accommodated', whereas the stomata of the rest of the leaf had closed as a result of the shock of detaching the cups from the leaf and the leaf from the plant.

Experiment 7 was undertaken to answer this objection. A plant was illuminated for 2 hours, and the alcoholic dye solution then poured on to a still-attached leaf, which was not otherwise touched in any way. No injection occurred. Since it is preferable that the dye solution should be poured on to the under side of the leaf, where the stomata are more numerous, a second plant was suspended upside down and similarly treated, with the same result. This experiment was repeated a number of times, and in no case was any evidence obtained that the stomata, on a leaf thus freely exposed to the air, ever open to the extent so easily demonstrated under a porometer-cup.

Experiment 8. In one replicate of expt. 6 it was observed that there had been slight injection elsewhere on the leaf than under the cup, and further examination showed that this injection followed almost exactly the line of the covering glass plate. This suggested that the phenomenon could be produced by some simpler form of enclosure than a porometer-cup. A glass plate was therefore supported horizontally just below a leaf so that about half the leaf was resting on the plate; a second glass plate was placed on top of the same half of the leaf and allowed to rest in position under its own weight. Approximately half the leaf was thus enclosed, though not hermetically, between two glass plates, the remaining half being freely exposed to the air. The leaf was illuminated for 2 hours and tested as before; the result is shown in Pl. VIII, 2, and it will be seen that the stomata on the enclosed half of the leaf have opened

as widely as under a porometer-cup. Similar experiments were carried out using a single glass plate, lightly appressed to either the upper or lower surface of the leaf. It was then found that the effect of applying the glass plate to the lower surface only was substantially equal to that produced by enclosure between two plates, but that a plate applied only to the upper surface had little effect, there being but slight injection.

4. Preliminary consideration of foregoing results

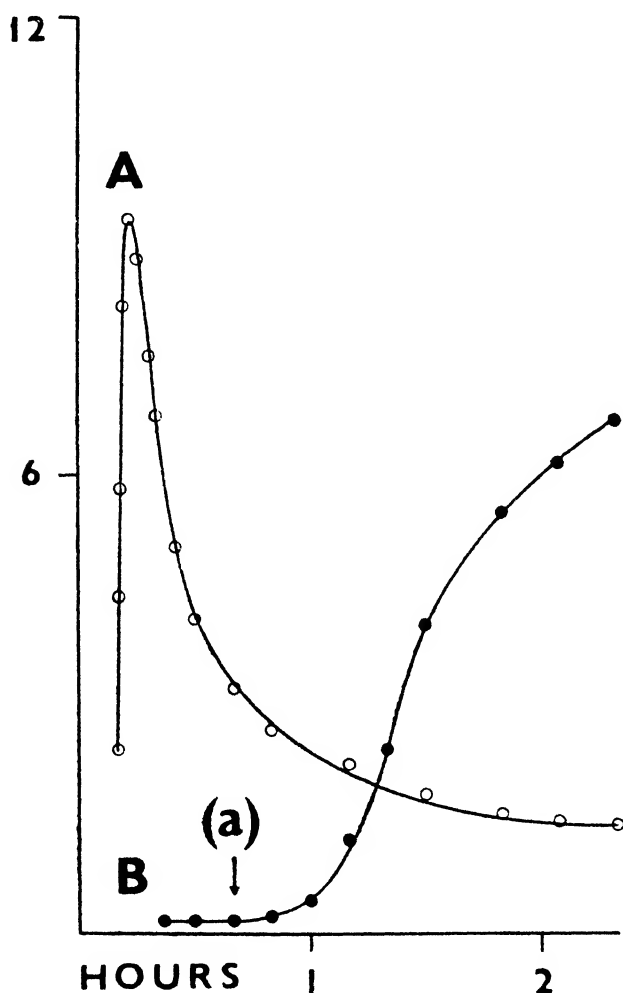
The conditions to which the leaf is exposed within an enclosure (porometer-cup or the space between two glass plates) differ in a number of respects from those to which it is exposed in free air; these differences have been set out by Heath and Williams (1948). The work of establishing which of these conditions was the primary cause of the phenomenon under investigation was undertaken by Heath, who in a preliminary report (Heath, 1948) has shown conclusively that it is due to the reduced CO_2 -content of the air in the enclosure. The wider implications will be considered at a later stage, but it will be convenient at this point briefly to examine the foregoing results in the light of Heath's discovery. In a porometer-cup set up in what is here regarded as the 'normal' manner, air is drawn slowly under the glass plate, across the ring of leaf-tissue above the gelatin washer, into the area immediately above the cup proper, and thence through the leaf. By the time the air passes out through the stomata of the lower epidermis, whether it has come by this route or laterally through the mesophyll, it may reasonably be supposed to be CO_2 -free; and the 'glass-plate' experiments suggest that it must be substantially free from CO_2 even by the time it enters the upper stomata. The irregular closure observed when the plate is removed is thus presumably due to the effect of ordinary laboratory air (normally regarded as containing *c.* 0.03 per cent. CO_2 by volume) on the *upper* stomata. The difference in behaviour between the leaves of the varieties *Paul Crampel* and *Salmon Crampel* is very interesting; if it is not due to an inherent difference in sensitivity, it may well be related to the difference in hairiness of the leaves. In completely still air the normal gas-exchange consequent on photosynthesis will no doubt result in there being a 'diffusion-shell' around the leaf, consisting of air of reduced CO_2 -content. However, the ubiquitous draughts and small air-movements to which a leaf is exposed even in a laboratory will tend to disperse this shell; and it might be expected that the less hairy a leaf, the more subject to dispersal will the shell be, and the more striking the 'glass-plate effect'. No specific investigations on these lines have been attempted, since no further plants of the variety *Salmon Crampel* have been available; but it may at least serve as a plausible explanation which avoids the postulation of varietal differences.

II. Cognate Phenomena

1. Supplementary observations

If the hypothesis now advanced is correct, two simple predictions may be made:

- (i) If the stomata are caused to open by enclosing part of the leaf between two glass plates, a porometer-cup then attached to that part of the leaf should show no Knight effect.



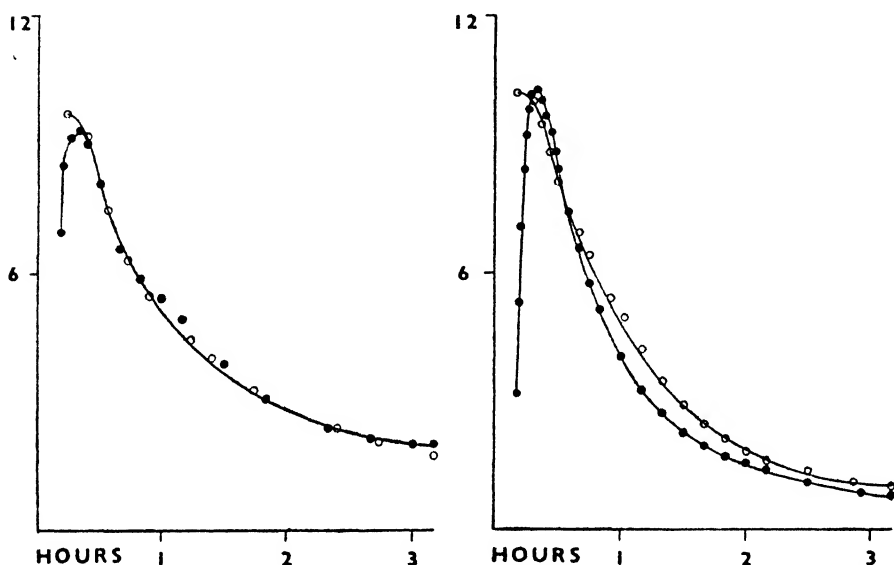
TEXT-FIG. 6. Showing absence of Knight effect when porometer-cup is attached to part of leaf previously enclosed between glass plates. See text, expt. 9.

- (ii) Since Knight's 'recovery' curve and any subsequent opening in light are not distinct phenomena, but merely successive examples of stomatal opening in CO_2 -free air, the curve obtained should be virtually identical in the two cases.

The following experiments illustrate these properties:

Experiment 9. Half of a leaf was enclosed between two glass plates as in expt. 8 and illuminated for 2 hours. A porometer-cup (record A in Text-fig. 6)

was then attached to the half *not* enclosed, and it will be observed that the normal Knight effect is shown. A few minutes later a second cup (record B in Text-fig. 6) was attached to the half which had until then been enclosed; there is no evidence of closure. At the point *a* on record B the second cup was darkened by placing a halfpenny on the glass plate over the cup; the stomata begin to execute a closing movement, thus demonstrating that the seal, which might otherwise have been suspect, was satisfactory.



TEXT-FIG. 7. Showing identity of 'recovery' curve with that of subsequent opening in light. Closed circles represent 'recovery' on first attachment of cup; open circles represent subsequent opening curve of same group of stomata in light.

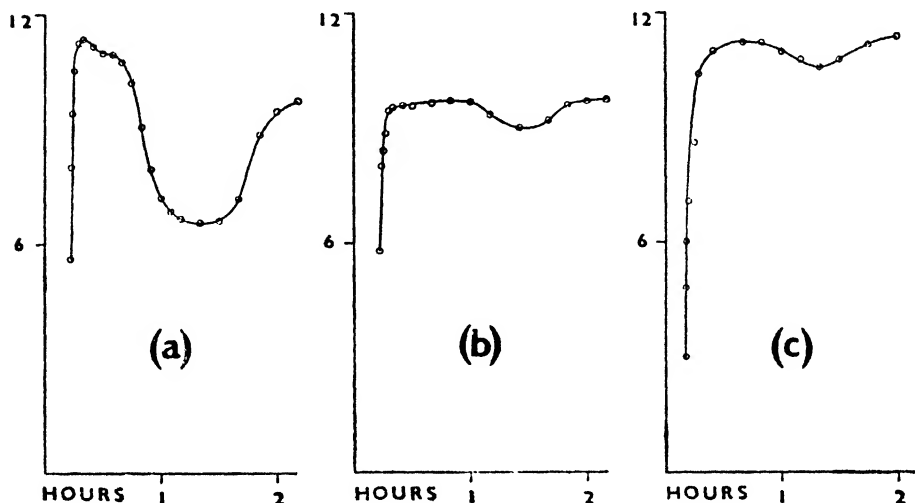
Experiment 10. A cup was attached and the Knight-effect curve obtained. The cup was then darkened (again by a halfpenny) until the resistance was of the same order as that attained at the peak of the Knight effect, then re-illuminated. The resulting opening curve, and the previous Knight-effect curve, were then plotted on the same graph. Two such double graphs are shown in Text-fig. 7, and it will be seen that, particularly in view of the known variability of stomatal movements, the closeness of fit of the two curves in each case is remarkable.

2. The Knight effect in darkness

Experiment 11. A cup was attached to an illuminated leaf in the ordinary way, and the cup immediately covered with a halfpenny, so that the cup (including the area of leaf over the washer), but not the rest of the sector or the leaf, was then in comparative darkness. Two examples of the resulting record are shown in Text-fig. 8(a) and (b); both show a closure, followed by a partial opening, then a return to the closing movement. This effect was

obtained on a number of occasions; the opening phase varied considerably in degree and in time of onset (usually about 30 minutes after fixing), but in only one case was it not shown at all.

An attractive explanation of this unexpected result could be elaborated along the following lines: At the moment of darkening both upper and lower stomata are appreciably open and a large part of the air-stream is passing vertically through the leaf; furthermore, at this point photosynthesis ceases, and the air passing through the leaf is ordinary laboratory air, enriched by



TEXT-FIG. 8 The Knight effect in darkness: (a) and (b), only the cup darkened, (c), the whole leaf darkened. See text, expts 11 and 12.

the CO_2 of respiration of the darkened area. The stomata consequently begin to close. However, as the stomatal aperture steadily decreases an increasing proportion of the air-stream is drawn *laterally* through the mesophyll, and this air, having passed through an illuminated portion of the leaf, is CO_2 -free. Now Heath, in his preliminary note (1948), states that CO_2 -free air will cause partial opening of stomata not already closed; this, then, provides a reasonable explanation of the opening. The further closing is less easy to explain, but may perhaps show that closure due to darkness will in any case supervene in time.

Experiment 12. If the above explanation is correct, then, if a porometer-cup is attached to an illuminated leaf and the *whole* leaf darkened, no source of CO_2 -free air is available, and there should be no partial opening. This experiment was therefore carried out, the whole plant being enclosed in a light-proof box and the light switched off immediately the cup had been fixed. The resulting record is shown in Text-fig. 8(c), and it will be seen that, contrary to expectation, the same closing-opening-closing cycle is obtained. (In view of the variation in degree of opening found in the replicates of

expt. 11, the reduced amplitude of opening is not necessarily significant.) A possible explanation of this disconcerting result is given in § III.

3. Genera other than *Pelargonium*

It is obviously of great importance to determine whether this sensitivity to CO₂-free air, with its attendant complications, is widespread or merely confined to a few genera. As mentioned in the preliminary notes, many of the experiments here described have been repeated by Heath on *Triticum vulgare* and found to hold good for that species. Knight's results, as mentioned in the Introduction, show that a similar effect is shown by species of *Eucharis*, *Ficus*, and *Begonia*; but he notes that in *Eucharis* and *Acalypha* shock-sensitivity is retained under a porometer-cup. (It may be stated here that such few observations as the author has made on *Eucharis mastersii* suggest that it was an unfortunate choice; it shows far less response to CO₂-free air than does *Pelargonium*, and all attempts to produce any appreciable effect by enclosure between glass plates have so far failed.) At the other end of the scale Heath (1938) reported that he was unable to obtain any evidence whatsoever of the existence of the Knight effect in *Cyclamen*. Lacking any detailed survey, therefore, it can only be stated that the Knight effect is undoubtedly shown by a number of unrelated genera of angiosperms, but that it cannot yet be assumed to be universal.

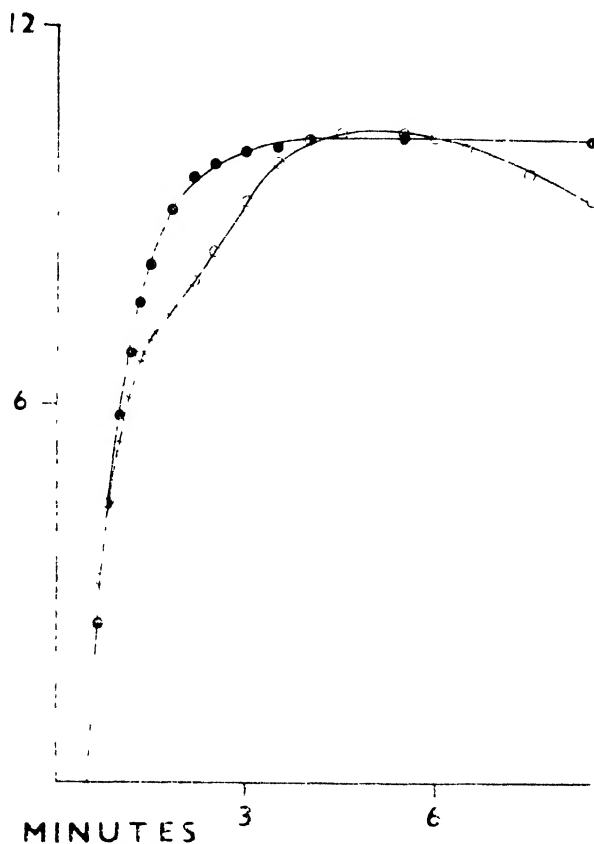
III. The Evidence for True Shock-sensitivity

Although the spectacular shock-effect postulated by Knight must now be discounted, it is clear from expts. 4 and 5 that, provided that they are not wide open, stomata do in fact respond to mechanical shock by a rapid temporary partial closure. It is therefore to be expected that such a response will also be given when a porometer-cup is first attached, but, as explained in the description of expt. 1, any such movement is obscured by the apparatus lag. However, a closer analysis of the beginning of the Knight-effect curve is revealing, as the results of the following experiment will show.

Experiment 13. A cup was attached in the normal way to an illuminated leaf, the manometer being read about every 10 seconds. The peak resistance attained by the stomata was noted, and the cup then replaced by a length of thermometer-tubing of as nearly this resistance as possible. The manometer was again read at approximately 10-second intervals and the two records thus obtained plotted on the same graph with an extended time-scale. The result is shown in Text-fig. 9. It will be seen that the porometer graph lags behind that of the thermometer tube, though attaining almost exactly the same peak value; this can only mean that during the lag period there is at first a rise in stomatal resistance. Evidently, then, there is a shock-response when the cup is attached, though of small magnitude.

A plausible explanation of this phenomenon can be given if it is assumed that stomata are sensitive to small increases, as well as decreases, in CO₂-content. We know from the experiments of Audus (1939) that a severe

mechanical shock causes an immediate rise in the respiration of a *Pelargonium* leaf by as much as 60 per cent., followed by a slow fall. When the stomata are nearly closed the porometer air-stream through the leaf is slow, and it may be that photosynthesis is insufficiently vigorous at the light-intensity employed—little more than 250 foot-candles as measured by a Weston light-



TEXT-FIG. 9. Demonstration of true shock-response on fitting a porometer-cup. Open circles, porometer-cup, closed circles, thermometer-tubing. See text, expt. 13.

meter—to dispose immediately of the increased CO_2 of respiration, so that there is a slight increase in the CO_2 -content of the air within the leaf. When the stomata are wide open, on the other hand, the relatively rapid stream of CO_2 -free air drawn through the leaf may be sufficient to keep the CO_2 concentration down to negligible proportions, despite the respiratory increase. A similar explanation may be advanced for the unexpected opening in darkness noted in expt. 12, the opening phase corresponding to the drop in respiratory CO_2 following the initial stimulation resulting from the attachment of the cup.

It must be admitted that these explanations are not completely convincing, but at least they do not conflict with known facts and may serve until further experiments can be undertaken.

DISCUSSION

There can be little doubt that detailed discussion of the phenomena here described must await the publication in full of Heath's researches into the effects of CO_2 -content. However, in view of the potential importance of these new observations, a brief consideration, if only to indicate possible repercussions, is desirable even at this early stage. The implications of the work can usefully be considered under four unrelated headings.

1. Sensitivity of stomata to mechanical shock

It is clear that stomata can no longer be regarded as structures profoundly sensitive, in the sense that *Mimosa* is, to mechanical shock, previous observations to this effect having been based on a misinterpretation of experimental data. However, some slight sensitivity can still be demonstrated, and it has been suggested in the foregoing that this response is an indirect effect resulting from the increase in respiratory CO_2 consequent on the shock. Whether this hypothesis is correct, only further work can decide.

2. The implications to porometer technique

Ever since Darwin (1898) remarked that 'This method requires elaborate apparatus and must, I imagine, be slow in action and difficult to manage, but I have no personal experience of it', the porometer has been a target for criticism. Darwin's specific objections have for long been inapplicable, the argument in recent years having rather concentrated on the possibility that the forced air-stream might distort the intercellular spaces of the leaf to an extent sufficient to invalidate the results obtained. It seems seldom, perhaps fortunately, to have occurred to the critics that stomata might behave abnormally under a porometer-cup, so that, though the porometer may reflect accurately the behaviour of the stomata actually under the cup, it provides no evidence of the state of the stomata on the remainder of the leaf. However, this is apparently the case with a porometer *as normally set up* on a plant which shows what has here been conveniently referred to as the 'Knight effect'. The present author does not wish to enter deeply into porometer theory; the precautions that should henceforth be taken in porometer work have already been briefly set out by Heath and Williams (1948) in which will be found an outline account of a method devised by Heath, using a positive-pressure aspirator and a cup through which air can be swept between readings to overcome the new difficulties.

3. The mechanism of stomatal movement

At first sight these results might appear to support the well-known hypothesis that stomatal movement is brought about by pH changes consequent on accumulation or removal of the CO_2 of respiration, an hypothesis which is especially associated with Scarth and his collaborators. In contrast, perusal of the work of the adherents of this hypothesis shows that the pH changes

under consideration are relatively enormous, covering approximately the range 4.5 to 7.0 units. However, the pH change consequent on the removal of 0.03 per cent. by volume of CO_2 in air must surely be far less than this, and a better analogy is perhaps to be found in mammalian respiration. It has for long been known that both the respiratory centre itself and the chemoreceptors of the carotid glomus are extraordinarily sensitive to very small variations in CO_2 -tension in the blood, changes equivalent to a pH change of no more than 0.02 units causing a marked alteration in ventilation rate. Perusal of a recent general account (Fulton, 1946) of the physiology of respiration suggests that there is at present no agreement as to whether the CO_2 acts specifically as an active molecule, or whether it acts via pH change; but further research into the mechanism of regulation of mammalian respiration may yet throw light on the similar remarkable sensitivity to CO_2 changes shown by the guard cells.

4. Biological significance

It has already been pointed out by Heath (1948) that this excessive sensitivity to CO_2 -content will probably result in the partial closure of stomata in wind, since in such circumstances the CO_2 -content at the surface of the leaf will be kept at its maximum; this would provide some degree of stomatal control of transpiration. The significance of hairiness will also need re-examination, since it seems likely that the stomata of a hairy leaf will be more consistently wide open than those of a glabrous one (cf. expts. 4 and 5 of this paper). It will be recalled that Sayre (1920) found that when the hairs on the leaves of *Verbascum thapsus* were removed, stomatal transpiration was but little affected; unfortunately, in this particular experiment he made no comparative examination of the stomata, and it is tempting to speculate that the removal of the hairs, while promoting increased transpiration, also caused partial stomatal closure as a result of the free exposure to air.

A further point which should be mentioned is the possibility that the wide opening might be regarded as an extreme case of 'CO₂-hunger'; if vegetation is so crowded that the CO_2 -content of the ambient air is inadequate to maintain growth, the very wide stomatal opening will at least enable such CO_2 as is available to be absorbed with the greatest possible efficiency.

In conclusion, it must be admitted that the results of this investigation have, if anything, complicated the stomatal situation. However, theories of stomatal movement have languished in recent years, largely owing to the absence of new information; and any new property, however inconvenient, is to be welcomed in the hope that it may in time contribute to a clearer understanding of stomatal mechanism.

SUMMARY

1. The 'Knight effect'—i.e. the apparent rapid closure of stomata in response to the attachment of a porometer-cup—is shown to be a misinterpretation. The porometer provides a substantially accurate measure of stomatal

aperture at the time of fixing, the subsequent 'recovery' being an abnormally wide opening due to the reduction in CO_2 -content of the air within the porometer-cup, and not occurring elsewhere on the leaf. Similar wide opening can be induced by enclosing the leaf, not necessarily hermetically, between two glass plates in the light.

2. There is, however, evidence of true response to mechanical shock, though of no great sensitivity; this is not shown when the stomata are in the condition of abnormally wide opening. It is suggested that this is an indirect effect due to a temporary increase in respiration as a result of the shock.

3. Certain cognate phenomena are described, and the evidence for the existence of the Knight effect in genera other than *Pelargonium* is briefly reviewed.

4. The implications of these findings are briefly discussed with reference to (a) porometry, (b) stomatal mechanism, and (c) biological significance.

ACKNOWLEDGEMENTS

It is with pleasure that the author records his indebtedness to Dr. O. V. S. Heath of Imperial College, not only for a crucial suggestion which has been acknowledged in its proper place in the text, but for the series of consultations and discussions which culminated in the joint preliminary account of this work; and to Professor F. G. Gregory, F.R.S., who participated in a number of these discussions. The author is also most grateful to Mr. G. Butler, of the Bedford College Botany Garden, for the care and skill that he has applied to the provision of the large number of *Pelargonium* plants needed for this investigation.

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EXPLANATION OF PLATE VIII.

Illustrating W. T. Williams's article 'Studies in Stomatal Behaviour III. The Sensitivity of Stomata to Mechanical Shock'.

FIG. 1. *Pelargonium* leaf to which two porometer-cups have been attached and allowed to 'recover' in light, after infiltration with alcohol. Injection is confined to the areas of the cups (Leaf photographed immediately after infiltration)

FIG. 2 *Pelargonium* leaf, part of which has been enclosed between two glass plates, after infiltration with alcoholic crystal violet. Injection is confined to the enclosed area. (Leaf dried and pressed before photographing)

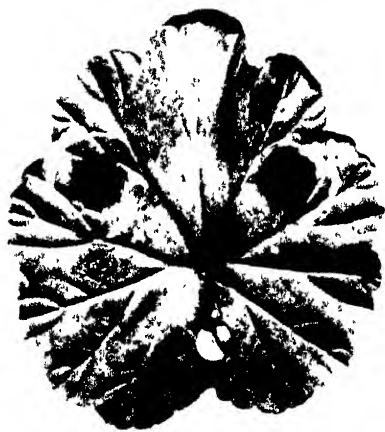


FIG. 1

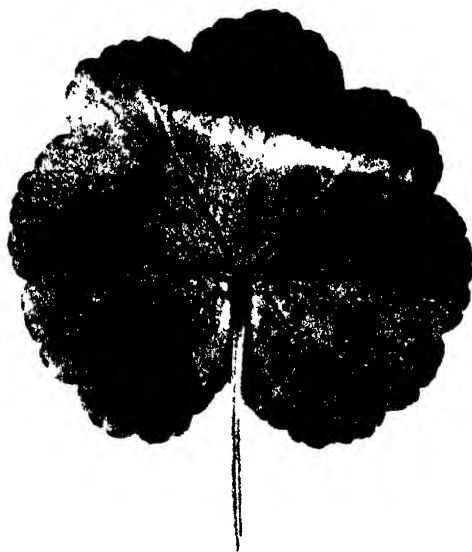


FIG. 2

WILLIAMS—STOMATAL BEHAVIOUR

The Effect of Isopropyl Phenyl Carbamate on Mitosis in Rye (*Secale cereale*) and Onion (*Allium cepa*)

BY

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With Plates IX and X

INTRODUCTION

IT was shown by Templeman and Sexton (1941, 1945) that ethyl phenyl carbamate exerted a selective effect on mixed stands of growing oats and charlock; the latter remained unaffected, but growth of the oats was seriously reduced. Morphological effects of the treatment on oats closely resembled those caused by colchicine, acenaphthene, and other known mitotic poisons, but were induced by much lower concentrations. Isopropyl phenyl carbamate behaved in a similar way to the ethyl compound but proved to be about three times as active. The present investigation was carried out to determine the cytological effects of isopropyl phenyl carbamate (I.P.P.C.) on the susceptible plants rye and onion.

METHODS

Solutions were prepared as follows:

The weighed amount of recrystallized I.P.P.C. was dissolved in 2-5 ml. of absolute alcohol. This concentrated alcoholic solution was then poured into 500 ml. of distilled water in a wide-mouthed bottle, the water being given a fast swirling motion as the alcoholic solution was poured in. The mixture was well shaken for a few minutes before being transferred to a graduated flask and made up to exactly 1 litre with distilled water. It was found to be essential that mixing of the alcoholic solution and distilled water be done very quickly, otherwise the I.P.P.C. separated out as crystalline particles easily visible to the naked eye. Mixtures to which this had happened were not used in any experiments.

The 200-p.p.m. solution closely approached saturation.

A corresponding amount of alcohol was added to the distilled water used for the controls.

(a) *Rye*. Seeds were germinated under Copenhagen jars on filter-paper moistened with distilled water; when root length reached 1-2 cm. the seeds were impaled on pins and suspended over the experimental solutions (I.P.P.C. at 0, 0.1, 1, 10, and 100 p.p.m.), only the roots being immersed in the liquid. Some fixations were made immediately after treatment, others 3-5 days later,

the seeds being kept under conditions similar to those during treatment but over distilled water during the intervening period.

(b) *Onion*. Bulbs were grown on 300-ml. conical flasks over distilled water until the roots reached a length of 1–2 cm., when they were transferred to flasks containing I.P.P.C. at 0, 50, 100, and 200 p.p.m., only the roots being immersed. Fixations were made immediately after treatment in all cases.

Two methods were used for the preparation of slides:

1. Root-tips of rye were fixed in Benda, embedded in paraffin wax (m.p. 52° C.), sectioned on a Cambridge rocking microtome at $10\text{--}12\ \mu$ and stained in Haidenhain's iron-alum-haematoxylin (20–4 hours staining after mordanting in 8 per cent. iron alum for $\frac{1}{2}$ –4 hours).
2. The Feulgen root-squash technique was used for both rye and onion. Fixation in this case was in CA 91 (1 per cent. chromic acid, 9 vols.; 5 per cent. acetic acid, 1 vol.), hydrolysis in N.HCl for 20 minutes at 60° C., and staining for $\frac{3}{4}$ –1 hour in leucobasic fuchsin, prepared by a modification of Coleman's method. Later preparations were counter-stained with fast green FCF or light green.

Preparations were examined by means of a Leitz microscope, using for critical work a $\frac{1}{2}$ -in. oil-immersion objective and $\times 10$ compensating eyepiece. A Leitz microscope lamp provided illumination, light being dimmed and reduced to suitable contrasting colour by means of green or orange filters. For some of the high polyploid cells a $\frac{1}{6}$ -in. dry objective provided adequate magnification.

Photographs were taken using the optical apparatus described above with camera extension of 11 in., giving a magnification of $\times 480$. Ilford Rapid Process Pan. or Kodak P.300 plates proved satisfactory.

RESULTS

Rye

The first preparations to be made (haematoxylin-stained sections) showed a series of mitotic irregularities. Daughter-chromosomes failed to separate at metaphase, and anaphase movement was apparently inhibited. In consequence, paired chromosomes were frequently to be seen (Pl. IX, Fig. 1) and polyploid restitution nuclei arose. These effects occurred after rates of treatment of 0.1–1.0 p.p.m. for 24 hours. After longer treatments at higher concentrations (e.g. 2 days at 10 p.p.m.) nuclei developed by repeated chromosome division without subsequent nuclear division into very high polyploids ($8n$, &c.) in which masses of chromosomes filled the entire cell (Pl. IX, Fig. 2). It appeared that chromosome division ceased to be synchronous at later stages of treatment, cells with irregular (aneuploid) chromosome numbers frequently occurring. After the maximum treatments (4–9 days at 10 p.p.m.) chromosomes sometimes ran together to form large, deeply staining, pycnotic masses (Pl. X, Fig. 2). The cells with high polyploid chromosome numbers were greatly enlarged in size and the walls were sometimes considerably thickened.

Death of the affected cells ensued, as was shown by preparations made 5 days after the completion of treatment for 4 days at 10 p.p.m., in which many enlarged but empty cells were to be seen (Pl. IX, Fig. 3). At certain stages bridges of chromatin could be seen at telophase or interphase, suggesting that chromatid breakage or interchange had taken place.

Later preparations, made from the same treatments by the Feulgen root-squash technique, confirmed these results and demonstrated clearly that chromatid fragmentation had occurred, acentric fragments being seen (Pl. IX, Fig. 4). Resting nuclei of irregular shape, often found in these preparations, suggested that spindle action had become abnormal before it was inhibited. The strain of rye used in these experiments was one in which fragments did not occur naturally, as was shown by the absence of fragments on slides prepared from control plants.

A feature of root-tips treated with I.P.P.C. at higher rates was that although both cell division and growth had been arrested, differentiation of cells immediately behind the meristematic region continued, with the result that fully developed tracheides and vessels were to be found within a few cells of the root cap.

Externally, the effect of treatment with I.P.P.C. was shown by the root-tips developing club-shaped swellings, characteristic of the action of mitotic poisons (the 'c-tumours' of Swedish cytologists). These were very marked after the highest treatments, when the root was sometimes represented by a small, almost spherical knob of tissue, 1-2 mm. in diameter, covered with root-hairs. Roots which had been transferred to water for 72 hours after treatment at 100 p.p.m. for 4 hours showed less drastic effects than those which had been kept in water for 5 days after treatment at 1 or 10 p.p.m. for 2-4 days. In the former, nuclear division was still taking place, there was evidence of polyploidy, and chromatid breakage and its effects were clearly seen. An abnormality not seen elsewhere was the presence in considerable numbers of enlarged nuclei apparently arrested at a late prophase, in which the nuclear membrane was very marked and contents consisted of prophase-type chromosomes, fragments or small pycnotic masses occupying a much smaller proportion of the nucleus than in normal prophase; large, apparently empty, unstained areas were to be seen in each nucleus.

Onion

The effects of I.P.P.C. on mitosis in the cells of the root-tip of the onion were clearly related to those seen in rye, but differed in a number of respects. These differences indicated that onion was very considerably less sensitive than rye to the action of the compound; consequently a given effect was produced only by much higher concentrations. Even at rates of up to 200 p.p.m. suppression of spindle action was less complete than in rye at 10 p.p.m., and consequently polyploid cells occurred in smaller numbers, none being higher than tetraploid (Pl. X, Fig. 5). Multipolar spindles occurred commonly at 50 p.p.m. and over (Pl. X, Fig. 6). Failure of the

daughter-chromosomes to separate at metaphase occurred at similar concentrations; the paired chromosomes were in many cases greatly contracted, especially at the higher rates of treatment (Pl. X, Fig. 7). Fragmentation was not clearly seen in the onion. There was evidence of nucleolar enlargement at the higher rates of treatment (100 p.p.m. and over).

Interphase nuclei of irregular shape—lobed, or consisting of several small, connected nuclei, apparently the result of multipolar spindles—also occurred in considerable numbers, as well as multinucleate cells (Pl. X, Fig. 8).

DISCUSSION

The effects of I.P.P.C. appear to resemble in many respects those due to colchicine and to a range of phenolic and amino-compounds (Levan and Tjio, 1948a). There are, however, certain deviations from the normal course of colchicine action in addition to effects not shown by colchicine. In some particulars also there is a similarity between conditions produced in plant cells by I.P.P.C. treatment and those found in many types of animal tumour.

Effects characteristic of colchicine (see Levan, 1938) include interference with centromere division, giving rise to typical paired chromosomes, and spindle suppression, causing polyploidy. These effects are to be seen in both rye and onion. Multipolar spindles, the resulting interphase nuclei of irregular shape and multinucleate cells, seen most frequently in the onion, are believed to be due to partial suppression of spindle action. They have been described as developing after colchicine-treated plants have been transferred to water and are recovering from the treatment. In the case of I.P.P.C. on onion, however, they occur during treatment. Over-contraction of chromosomes, generally associated with colchicine treatment, has been observed in the onion after the higher rates of I.P.P.C. treatment (50 p.p.m. and over) and occasionally in rye; in all other cases chromosomes showed the normal degree of metaphase contraction, as seen in controls. These effects are similar to those described by W. B. Ennis, jr. (1948) in oat and in onion treated with I.P.P.C.

An effect of I.P.P.C. not found after colchicine treatment is the fragmentation of chromatids; acentric fragments have been seen in several nuclei of rye (Pl. IX, Fig. 4). It is here that the resemblance to tumours is noticeable, as fragmentation of chromosomes and polyploidy are associated in many types of neoplastic growth (Koller, 1947b). Though fragments have been seen fairly frequently in rye, their occurrence in onion, even after the highest rates of I.P.P.C. treatment, remains doubtful. Fragmentation was not reported by W. B. Ennis, jr., in onion or in oats after I.P.P.C. treatment.

It is difficult to decide at what point in the mitotic cycle fragmentation has taken place, but one instance at least suggests that it occurs later than after X-ray (Lea, 1946), mustard gas (Koller, 1947b), or 2-methyl-4-chloro-phenoxy acetic acid (Doxey and Rhodes, 1948) treatment. The fragment in Pl. IX, Fig. 4, can be seen lying close to the chromatid from which it was apparently derived, suggesting that breakage had taken place shortly before fixation,

i.e. at late prophase or early metaphase, unless normal movement of chromosomes in the cell at these stages has been prevented.

A similar tentative conclusion with regard to time of breakage has been reached by Levan and Tjio (1948*b*) in their work on the action of phenolic compounds on mitosis. The present case differs from theirs, however, in that chromosome breakage is associated with polyploidy, whereas in theirs the two effects almost invariably occur at different concentration levels.

The cause of chromosome fragmentation remains obscure; in order that breakage might take place the basic polypeptide chain must have been attacked. In other cases (mustard gas, &c.) it is considered probable that breakage has taken place during interphase or early prophase, when the protective 'charge' of nucleic acid is at a minimum. If breakage after I.P.P.C. treatment proves to be at a later stage in mitosis, an alternative explanation must be found for the present case.

There is no visible evidence of corrosion of the chromatic substance of the chromosomes after I.P.P.C. treatment, such as was reported by Levan and Tjio (1948*a*).

Koller (1947*a*) has suggested that mitotic abnormalities prevailing in tumours are due to shortage of food and to toxic breakdown products of the neoplastic tissues. In the present case it does not seem likely that the food-supply to cells showing mitotic abnormalities is affected, the root structure being apparently normal. The effects are more likely to be due to the toxic properties of the compound itself, acting either by competing for an essential metabolic substrate, or by chemical action on the enzymes connected with spindle formation or on the respiratory enzymes essential for the process. This mode of action would correspond with the 'direct chemical action' of Levan and Tjio (1948*b*), as opposed to the theory of physical action put forward by Östergren (1944).

Dustin (1947) has considered the action of compounds affecting mitosis and suggests that carbamates interfere with purine metabolism; this would presumably affect the nucleic acid cycle of the cell. Some slight evidence for this is provided by the present investigation, e.g. the enlarged nucleoli and over-contracted chromosomes seen in onion root-tips after treatment at the higher rates.

As mitotic disturbances appear at lower concentrations, however, and are then unaccompanied by enlarged nucleoli or other evidence of disturbed nucleic acid metabolism, it does not appear that interference with nucleic acid metabolism is a primary or major cause of the mitotic irregularities.

The action of I.P.P.C. may now be considered in relation to 'endomitosis' and to the 'Spinacia effect' (Gentcheff and Gustafsson, 1939), in which chromosomes appear already paired at early metaphase and then undergo normal division; an additional chromosome division appears to have taken place during interphase. A similar effect has been reported as a result of treatment with growth hormones (Levan, 1939; Gentcheff and Gustafsson, 1939). In I.P.P.C.-treated roots metaphase chromosomes are often seen

to be single; it therefore appears that the I.P.P.C. effect is not similar to induced 'endomitosis' or to that seen in *Spinacia* (untreated plants), but is more closely related to the colchicine effect.

Finally, the relationship of these cytological effects to growth reduction must be considered. At the higher rates of treatment (i.e. 10 p.p.m. in rye or 100–200 p.p.m. in onion) suppression of cell division would be sufficient to prevent growth; at lower rates (i.e. 0.1–1 p.p.m. in rye and 50 p.p.m. in onion) rate of cell division is reduced and growth rate is likely to be reduced also. Although cell division may be prevented when polyploidy develops, cell enlargement continues, though possibly not in proportion to increase in chromosome number. (This is confirmed by the overcrowded condition of such high polyploid cells.) Differentiation found close to the root-tip shortly after or even during I.P.P.C. treatment suggests that either this process continues even after growth has been suppressed or perhaps that a change in activities from growth to differentiation has taken place in the cells. Such a change might well be due to interference in the nutrition of the cells, caused either by blockage of the supply of nutrients or to prevention of utilization of nutrients available in the cell by the action of the I.P.P.C. The change from cell division to differentiation would obviously be accompanied by a reduction or suppression of growth of the whole root.

SUMMARY

Growing roots of rye and onion were treated with isopropyl phenyl carbamate at 0.1–200 p.p.m. for 4–44 hours. Root-tips were fixed on completion of treatment or 3–5 days later, the roots being kept in distilled water during this period.

Effects described include interference with centromere action and spindle suppression, resulting in paired chromosomes and polyploid nuclei, in both rye and onion. Multipolar spindles and the consequent multinucleate cells occurred in the onion only, while chromatid fragmentation took place only in rye.

The effects are compared with those of colchicine and other mitotic poisons and with conditions prevailing in certain types of tumour.

ACKNOWLEDGEMENTS

The author would like to express his gratitude to Dr. W. G. Templeman for drawing his attention to this problem and for constant interest and helpful criticism, and to Miss S. M. Cooke for her assistance in preparing slides and photographs.

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DESCRIPTION OF PLATES

Illustrating D. Dovey's article 'The Effect of Isopropyl and Phenyl Carbamate on Mitosis in Rye (*Secale cereale*) and Onion (*Allium cepa*)'.

PLATE IX

Figs. 1-4 Rye

Fig. 1. Paired chromosomes (1,000). The chromosomes have divided, but, owing to suppression of spindle activity, have failed to separate. In some cases the centromere has not yet divided.

Fig. 2. High polyploid cell containing over 80 chromosomes, and pycnotic degeneration of similar cells (1,000).

Fig. 3. Large, empty cells, some with thickened walls (500).

Fig. 4. Chromatid fragmentation (1,000).

PLATE X

Figs. 5-8 Onion (All 1,000)

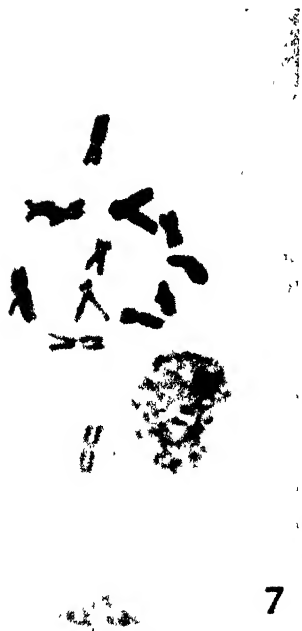
Fig. 5. Tetraploid cell (32 chromosomes)

Fig. 6. Multipolar spindle

Fig. 7. Paired chromosomes, greatly contracted. The centromere has divided in some but not in others, which show characteristic 'v' formation ('c-pairs').

Fig. 8. Multinucleate cell at interphase, and telophase resulting from irregular spindle activity.





Studies in the Physiology of Obligate Parasitism

III. The Growth of Rust Mycelium out of Infected Leaves

BY

SYDNEY DICKINSON

(School of Agriculture, Cambridge)

With one Figure in the Text

INTRODUCTION

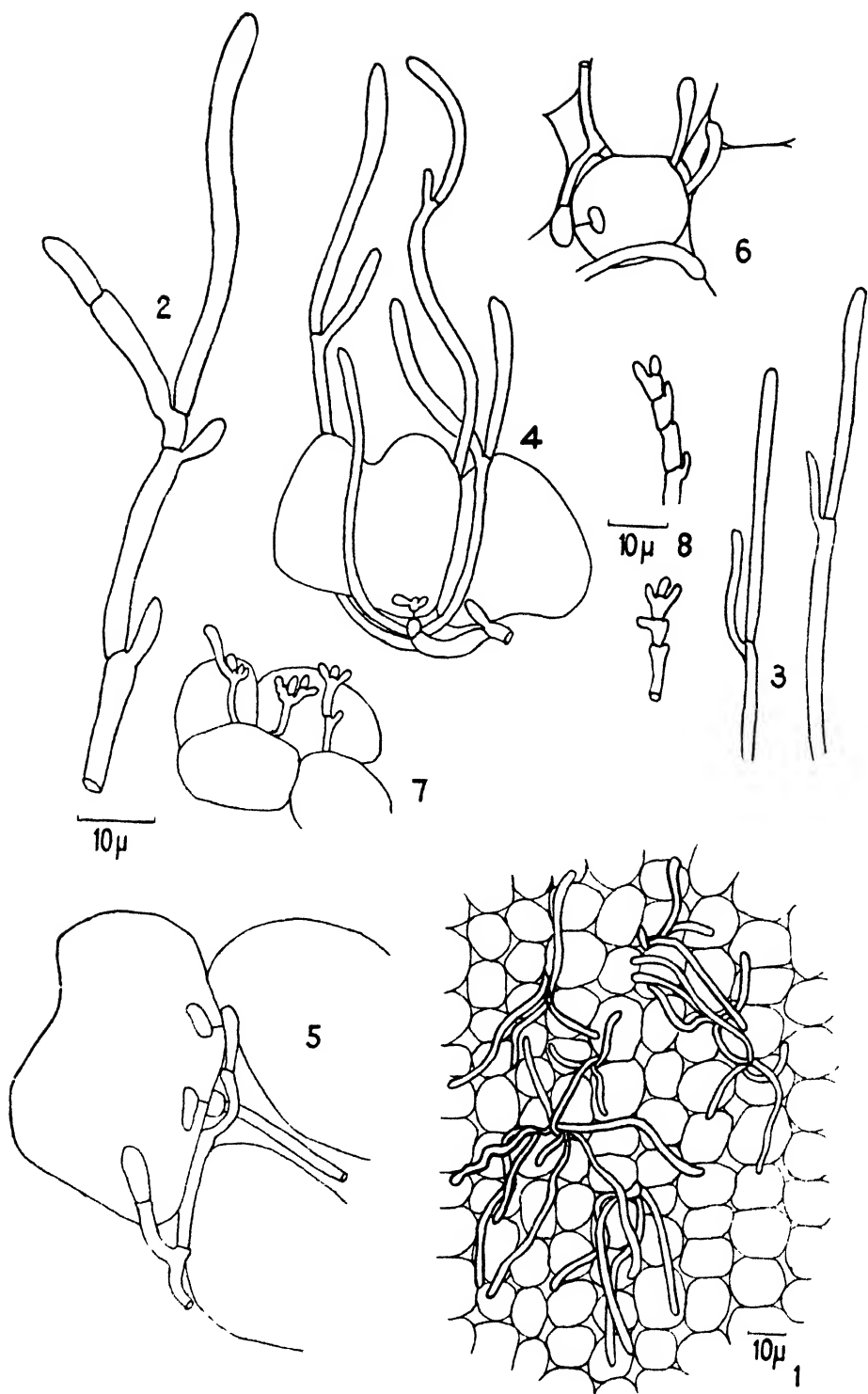
THE inoculum used for making fungal cultures on artificial media normally consists either of spores or of hyphae which have grown out of infected tissues. Hitherto no growth of a mycelium out of host tissues parasitized by rusts has been reported, with the possible exception of that observed by Falck (1935). He described a 'conidial form of growth' from pine wood infected with *Peridermium pini*. The result of the failure to obtain hyphae from infected tissues has meant that spores only have been used as the source of inoculum in attempts to grow rusts on artificial media. The following experiments show that hyphae will grow out of leaf tissues parasitized by *Puccinia triticina* and *P. graminis*.

Drawings illustrating the results are given in the Text-figure, these being made with a camera lucida using a $\frac{1}{8}$ -inch objective. The preparations were stained in cotton blue and lactophenol and mounted in lactic acid-glycerine.

EXPERIMENTS

Following on the failure to obtain septa formation in the germ-tubes of uredospores (Dickinson, 1949), an examination was made of the process of haustorial formation in the living wheat leaf. This was done by removing the lower epidermis from pieces of infected leaves which were then placed in ordinary Van Tieghem drop cells. The infected mesophyll so exposed was kept under constant observation for some days. The description by Allen (1926) of haustorial formation was confirmed. In addition, tufts of long and straight hyphae were sometimes to be seen growing out from the mesophyll (Fig., 1). Their branches, which were rarely formed, always came from immediately below a septum (Fig., 1, 2, 3). The frequent and characteristic appearance of these tufts, together with the form of their hyphae, suggested that they arose from the rust mycelium. The following experiments were made to test this suggestion.

Leaves of young susceptible wheat plants, which were just showing the flecking stage of infection, were cut off and put in a humidity chamber (R.H. c. 98 per cent.). While in this chamber a Boradaile needle was inserted



TEXT-FIGURE (see opposite).

in the lower epidermis, thus separating it from the mesophyll. In withdrawing the needle the lower epidermis was cut through and left as a flap. This was now gripped by a pair of forceps, and by careful pulling, a large area of mesophyll tissue was exposed; frequently the whole under-epidermis of a leaf was removed in one operation. The stripped leaves were cut into lengths of approximately 0.75 cm., and placed with the exposed mesophyll upwards on 3 per cent. gelatin containing 7.5 per cent. sucrose in Van Tieghem drop cells. A plain collodion membrane with distilled water on the upper surface was used instead of a coverslip. With experience it was found possible to distinguish the presence of hyphal tufts with the naked eye. They gave to the exposed mesophyll infected with *P. triticina* a slightly blue tint and a furry appearance. In *P. graminis* the slightly dirty-white mycelial tufts up to a millimetre in height were easily seen. After 48 hours all the leaf-strips were stained with cotton blue dissolved in lactophenol. On microscopical examination (see Table I) numerous haustoria of the usual rust form were found in the underlying mesophyll of all the strips bearing hyphal tufts (Fig., 4, 5, 6).

TABLE I

Occurrence of Hyphal Tufts and the Presence of Rust Haustoria in Strips of Infected Leaves at the Flecking Stage, placed in Modified Van Tieghem Drop Cells on 3 per cent. Gelatin 7.5 per cent. Sucrose

	Total number of leaf-strips.	Number of leaf- strips showing hyphal tufts, as determined under the microscope after 48 hours.	Number of leaf- strips showing haustoria.
<i>P. triticina</i> .			
Infected leaves	253	251	251
Uninfected leaves	46	0	0
Unstripped infected leaves	50	0	50
Unstripped uninfected leaves	50	0	0
<i>P. graminis</i>			
Infected leaves	79	75	77*
Uninfected leaves	25	0	0
Unstripped infected leaves	50	0	50
Unstripped uninfected leaves	50	0	0

* Probably due to the exposed mesophyll having come in contact with the gelatin.

TEXT-FIGURE. The drawings in this figure have been made with the aid of a camera lucida, using a Spencer Lens microscope and a $\frac{1}{4}$ in. objective. The eyepieces used were $\times 6$, $\times 8$, and $\times 15$. All preparations were stained in cotton blue and lactophenol and were mounted in lactic acid-glycerine.

1. Hyphae of *P. triticina* growing out from the infected mesophyll tissue of a leaf. 2. Hyphae of *P. triticina* showing characteristic branching. 3. Hyphae of *P. graminis* showing characteristic branching. 4. Hyphae of *P. triticina* growing away from infected mesophyll cells, one of which contains a haustorium. 5. Mesophyll cells, infected with *P. triticina*, showing haustoria and their parent hyphae. 6. Mesophyll cells, infected with *P. graminis*, showing haustoria and their parent hyphae. 7. Hyphae growing from uredospore initial hyphae of *P. triticina*. 8. Hyphae growing from uredospore initial hyphae of *P. graminis*.

The following experiment was made to demonstrate that the hyphae of the tufts were those of *P. triticina* or *P. graminis*. Leaf-strips bearing hyphal tufts were placed, tufts inwards, on exposed mesophyll of uninfected leaves of wheat seedlings. They were fastened in place by a collodion membrane wrapped round the leaves. The pots of wheat seedlings were then put into a tray of water and covered with a bell-jar. After 48 hours the leaf-strips were removed and the exposed mesophyll of the plants covered with a piece of wax-collodion membrane which prevented drying out. If uredosoral initials were found in the removed strips, the seedlings were discarded. Control experiments were made as shown in Table II. Successful infection of such leaves

TABLE II

Effect of inoculating the Mesophyll of Leaves of Wheat Plants with Leaf-strips bearing Hyphal Tufts

Inoculum.	Treatment of leaves.	<i>P. triticina</i> .		<i>P. graminis</i> .	
		Number of leaves treated.	Number of leaves developing rust sori and spores	Number of leaves treated.	Number of leaves developing rust sori and spores.
Leaf-strips showing hyphal tufts	Stripped (mesophyll exposed)	94	59 (62%)	42	29 (69%)
Leaf-strips showing hyphal tufts	Unstripped	24	0	21	0
Leaf-strips from uninfected leaves	Stripped (mesophyll exposed)	20	0	20	0
Leaf-strips from uninfected leaves	Unstripped	20	0	20	0

(i.e. the formation of uredospores) was obtained in 62 per cent. and 69 per cent. of the seedlings. It was concluded that the tufts of hyphae growing from the exposed mesophyll of rust-infected leaves were composed of rust hyphae.

Experiments were made (see Tables III A and III B) to determine how soon after infection mycelial tufts appeared. Control experiments were made with both uninfected leaves and unstripped leaves, and it is noteworthy that hyphal tufts were never observed arising from unstripped leaves. The length of leaf used was approximately $\frac{1}{4}$ in., and all leaves were infected on the same day from the same source of spores. The numbers obtained showed an increase in the number of leaf-strips with hyphal tufts until almost all strips showed such tufts; this occurred when flecking was visible in the leaves. Up till this stage the size of the tufts had steadily increased. As the uredosoral initials became visible, short and much-branched hyphae appeared in the central part of the hyphal tufts (Fig., 7, 8), though round them there was still a ring of long, unbranched hyphae. When the uredosorus was actually forming spores, few or no long hyphae developed; the short, much-branched hyphae were apparently the uredospore-forming branches.

A series of leaf-strips, infected with *P. triticina* at the flecking stage, were then made and placed on different media in Petri Dishes. The stripping was

TABLE IIIA

Effect of Age of Infection on the Outgrowth of Rust Hyphae from Exposed Mesophyll in Leaf-strips, approximately $\frac{1}{4}$ in. long. The Leaf-strips were placed on 3 per cent. Gelatin 7.5 per cent. Sucrose in Petri Dishes, and examined after 48 Hours. Greenhouse Temperature: average 58° F.

P. triticina.

Age of infection in days.		Stripped infected leaves.	Unstripped infected leaves.	Stripped uninfected leaves.	Unstripped uninfected leaves.
0	No. of strips used	58	25	36	25
	% showing hyphal tufts	0	0	0	0
3	No. of strips used	56	25	42	25
	% showing hyphal tufts	44	0	0	0
6	No. of strips used	57	25	41	25
	% showing hyphal tufts	68	0	0	0
9	No. of strips used	47	25	52	25
	% showing hyphal tufts	83	0	0	0
12	No. of strips used	51	25	45	25
	% showing hyphal tufts	100	0	0	0

(flecking was visible on the 9th day)

TABLE IIIB

Effect of Age of Infection on the Outgrowth of Rust Hyphae from Exposed Mesophyll in Leaf-strips approximately $\frac{1}{4}$ in. long. The Leaf-strips were placed on 3 per cent. Gelatin 7.5 per cent. Sucrose in Petri Dishes, and examined after 48 Hours. Greenhouse Temperature: average 58° F.

P. graminis.

Age of infection in days.		Stripped infected leaves.	Unstripped infected leaves.	Stripped uninfected leaves.	Unstripped uninfected leaves.
0	No. of strips used	31	25	40	25
	% showing hyphal tufts	0	0	0	0
3	No. of strips used	49	25	52	25
	% showing hyphal tufts	0	0	0	0
6	No. of strips used	39	25	42	25
	% showing hyphal tufts	5	0	0	0

TABLE IIIB (continued)

		<i>P. graminis.</i>			
Age of infection in days.		Stripped infected leaves.	Unstripped infected leaves.	Stripped uninfected leaves.	Unstripped uninfected leaves.
9	No. of strips used	48	25	49	25
	% showing hyphal tufts	35	0	0	0
12	No. of strips used	52	25	39	25
	% showing hyphal tufts	42	0	0	0
15	No. of strips used	53	25	41	25
	% showing hyphal tufts	58	0	0	0
18	No. of strips used	52	25	38	25
	% showing hyphal tufts	(flecking was visible on the 18th day)			
21	No. of strips used	45	25	41	25
	% showing hyphal tufts	96	0	0	0

carried out as before in a humidity chamber. From Table IV it will be seen that the type of medium had no effect on the appearance of the hyphal tufts, but the number of hyphae in the tufts and their length was affected by the concentration of sucrose.

TABLE IV

Effect of Different Media on the Formation of Hyphal Tufts of P. tritici by the Exposed Mesophyll of Leaf-strips, approximately $\frac{1}{4}$ in. long. The Age of Infection of the Leaves was 8 Days, and the Observations were made after 48 Hours

Medium.	Number of leaf-strips used.	Number showing hyphal tufts.	Indication of number of hyphae in tufts.	Approximate length of hyphae in tufts.
2% agar	25	24	×	50 μ
Potato dextrose agar	25	25	× × ×	300 μ
Dox's agar	25	25	✓ ×	200 μ
Malt agar	25	25	× ×	200 μ
Oatmeal agar	25	25	× ×	200 μ
3% gelatin	25	25	×	50 μ
3% gelatin plus 2.5% sucrose	26	25	× ×	100-150 μ
5% „	25	25	× × ×	200-300 μ
7.5% „	25	25	× × × ×	300-400 μ
10% „	27	27	× × × × ×	500 μ

DISCUSSION

Experiments demonstrate that hyphae of the two species of *Puccinia* used will grow out of infected host tissues on removal of the epidermis. Their failure to do so from uninjured leaves might be due to lack of sufficient power to break through the epidermis. It may be that the cell-walls had not been weakened by enzymes from the rust fungus or that the hydrotropic responses of the leaf hyphae were different from those of the germ-tubes.

The characteristic branching of the hyphae from below a septum was similar to the growth of the mycelium in leaf tissues as described by Allen (1926). The comparative scarcity of these branches, which perhaps was due to the lack of the usual contact stimulus given by the mesophyll cells, made the tufts distinctive.

It was probable that the number of hyphal tufts obtained during the early period of incubation would have been larger if more than 48 hours had been allowed. The difference in the number of tufts produced by the two rust species was correlated with the longer incubation period of *P. graminis*, and presumably with its slower growth at the temperature employed. The greater vigour of growth, as indicated by the larger numbers of hyphae and their greater length, observed on the media containing a higher sucrose content may be parallel to the results reported by Mains (1917). He obtained normal growth of rust on plants, kept in the dark and fed on sucrose. He also observed (Mains, 1915) a greater length of germ-tube on sucrose solutions than on distilled water.

SUMMARY

Experiments are described which show that on removal of the epidermis, the mycelium of *Puccinia triticina* and *P. graminis* will grow out as tufts of hyphae from infected leaf tissues.

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Studies in the Physiology of Obligate Parasitism

IV. The Formation on Membranes of Haustoria by Rust hyphae and Powdery Mildew Germ-tubes

BY

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With one Figure in the Text

INTRODUCTION

CONFLICTING opinions have been published on the role of the haustoria in the nutrition of obligate parasites. For example, Rice (1927) mentioned species in which abundant mycelium is present in spite of a late or sparse appearance of haustoria. She listed examples from all the larger groups of the Fungi which formed so-called haustoria in the host and which can be grown on artificial media. She therefore questioned whether haustoria are essential for food absorption. Brown (1936) thought that an obligate parasite might require nitrogen in a fully elaborated protein form as well as some growth substances. He further suggested that the haustorium was essential because it was responsible for food absorption. In view of this conflict of opinion it seems likely that the role of haustoria in nutrition cannot be determined until they have been produced apart from the host plant.

From Allen's (1926) description of the process of haustorial formation in *Puccinia triticina*, she seems to consider their formation as a reaction to contact with the walls of the mesophyll cells. Previously (Dickinson, II, 1949) it has been shown that the appressorial and substomatal vesicle formation in three species of *Puccinia* was a contact reaction. These observations, together with the subsequent evolution of a method of obtaining a plentiful supply of rust hyphae from infected leaves (Dickinson, III, 1949), have led to the present study of haustorial formation.

Contact reactions in the mildews have been investigated by Neger (1902) and Corner (1935), both of whom showed that the formation of the characteristically club-shaped appressorium is the reaction to a contact stimulus. The importance of the cuticle was emphasized by Graf Marin (1934), who showed that its removal from old resistant barley leaves rendered them susceptible to attack by *Erysiphe graminis*. The form of the haustorium was described by Grant Smith (1900).

The work of Hawkins and Harvey (1919), Brown and Harvey (1927), and Brown (1915) on the penetration of cell-walls by fungal hyphae has shown that this process in some fungi is a combination of mechanical penetration

and pre-softening by enzymes. Before the wall is penetrated the hyphal tip has to be firmly anchored. In many fungi a mucilaginous tip has been observed and indicated as the means of adhesion. Its absence from the apices of the germ-tubes of rusts and mildews has been noted (Dickinson, II, 1949; Corner, 1935).

METHODS

The method used to obtain hyphae from a leaf infected with *P. triticina* was to lay the freshly stripped mesophyll of a 4–7-day-old infected leaf on a membrane on the surface of 4 per cent. gelatin. The exposed mesophyll was thus in contact with or adjacent to the membrane. The leaf strip was carefully lifted off 48 hours later and the membrane examined microscopically. It was necessary to use semi-solid gelatin in order to increase the adhesion between the membrane and the material below it; if liquid was used, the membrane was very liable to tear. With semi-solid gelatin the adherent hyphae broke close to the mesophyll cells. It should be emphasized that the only substances used below the membranes in all experiments described have been 4 per cent. gelatin or distilled water.

The method of making collodion membranes has been described (Dickinson, I, 1949). The solution used contained 0.2 per cent. collodion in 9/91: alcohol/ether, with 0.075 per cent. paraffin wax of the required congealing-point.

The gelatin membranes were made by drying down a known volume of 1.5 per cent. solution on carefully levelled, polished, dental-rubber sheeting; SO₂ was used to prevent bacterial growth. After drying, the gelatin sheet was separated from the rubber by gentle pulling, and cut to the required size. Membranes were produced by this method down to a thickness calculated to be 20 μ . They were hardened in the required strength of alcohol formalin for 24 hours, thoroughly washed in distilled water, and then dried. The paraffin wax was deposited by flooding the membranes, lying on clean mercury, with a solution of wax in ether, and allowing the ether to evaporate. By this means a double membrane, paraffin wax overlying gelatin, was formed.

EXPERIMENTS WITH *PUCCINIA TRITICINA* ERIKSS.

Examination of membranes which had been in contact with exposed, infected leaf mesophyll for 48 hours showed clusters of hyphae up to about 200 μ in length. On removal of the leaf mesophyll the break between the adherent hyphae and the parent mycelium took place near the latter and was usually marked by a small globule of liquid, coming from the cell or cells ruptured in the break. Once this break occurred, there was no further growth on the semi-solid gelatin or on distilled water.¹ Therefore their development could only be followed with the leaf mesophyll *in situ* and under a low-power lens. Before contact with a membrane the hyphae were non-septate and unbranched. After contact they grew along and seldom away from the mem-

¹ The same result was obtained on the usual laboratory culture solutions.

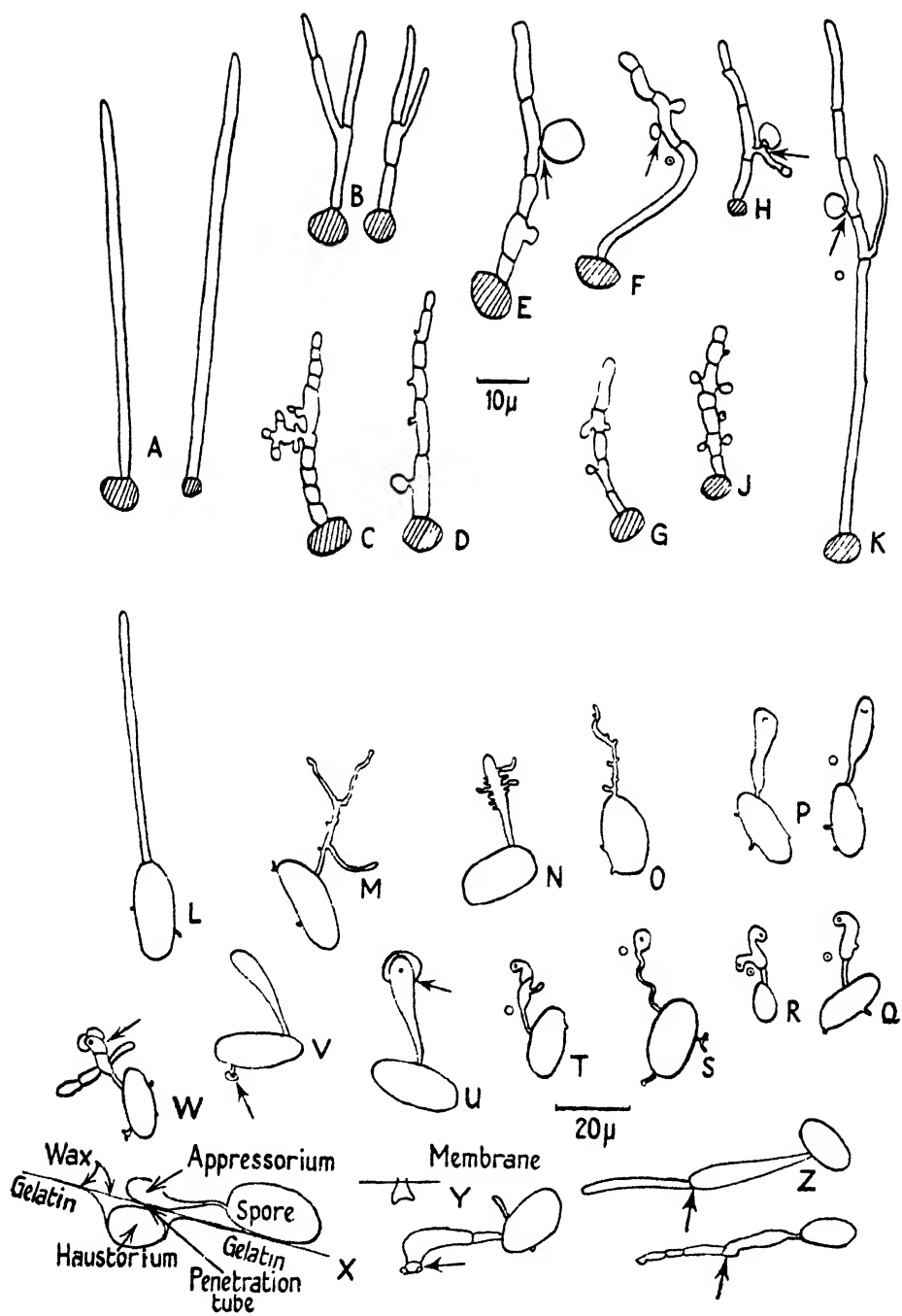
brane. No bursting of the hyphae or red granular coloration or increase in diameter has been seen before or after contact. This contrasts strongly with the behaviour of the germ-tubes on the same membranes (Dickinson, II, 1949).

The form of the hyphae and their branches are illustrated in the Text-figure (*a-k*). The simplest hyphae were relatively unbranched and non-septate, with small diameters (*a*). When branches were formed they always came from the apex of a cell and grew nearly parallel to the parent hypha and in the same direction (*b*). The membranes on which they grew were made from a series of paraffin waxes (congealing-points 36°C. – $65/71^{\circ}\text{C.}$) in a large number of different batches of collodion. In some cases there was an increase in the branching and septation, and the hyphal tip was more rounded than the usual tapered tip of the unbranched hypha (*c*). Septation was not always associated with branching and some of the branches were squat; this was particularly the case when the parent infection was old and nearing spore production. Probably this increased branching and septation was associated with spore production (Dickinson, III, 1949).

When BDH collodion (dated 1936) and the lower congealing-point waxes were used, more septation occurred without any accompanying branching. Instead, structures of a different kind with short and rounded ends were produced towards the middle of the cells of such septate hyphae (*g, j*). They grew out at right angles to the parent hyphae and in the plane of the membrane. None has been seen growing from the sides of the hyphae adherent to or farthest away from the membrane. They first appeared on the side of the hypha just above the point of contact with the membrane. Sometimes two were formed by one cell and they were then usually on opposite sides. Their stalks were short with reduced diameter and showed a septum across the narrowest part. Their adhesion to the membrane was considerable, as tested by a glass rod. There was as a rule a small black spot or ring in the centre of the structure (*j*), and focusing showed this spot to be on the side adhering to the membrane. The appearance of these black spots or rings was similar to those seen in *E. graminis* (see later), in which it is suggested they were due to air pockets at or around points of attempted penetration.¹

With BDH collodion (dated 1936) and the higher congealing-point waxes in the membranes these structures were considerably larger in size, being more rounded and 10 to 20 μ in diameter (*e*). Only their later stages of development and penetration have been followed. As stated earlier, they were rather oblong with a defined stalk (*d*), the black spot or ring being always present. No further change occurred in the stalk, but the structure itself gradually swelled and at the same time focusing showed it to be depressing the membrane (*f*). Soon after the oblong form had become a round one the black spot or ring disappeared. By this time the radiating strain lines indicated the depression of the membrane. As a rule these lines did not extend beyond the farther side of the parent hypha. Further swelling and depression of the membrane occurred until suddenly a slight crack appeared in it; at once the

¹ Repeated tests with indian ink showed no sign of mucilage on the structure of hyphal wall.



TEXT-FIGURE (see opposite).

membrane moved up round the structure until the depression disappeared (*e, h, k*). The split in the membrane showed towards the base of the now fully rounded structure as a crack or two stretching out a short distance. No further development has been observed even on membranes left in contact with the infected leaf mesophyll for 6 days rather than the usual 2. Cytological preparations have confirmed the penetration of the membrane and shown that two or more nuclei were present both in the structures and in each cell of the parent hypha.

EXPERIMENTS WITH *ERYSIPIE GRAMINIS* DC.

In the preceding experiments the inoculum was rust hyphae from an infected leaf. This was used because further growth on membranes had not been obtained from uredospores (Dickinson, II, 1949). The similar experiments with mildew spores will now be described.

On the germination of a mildew spore, usually one, sometimes more, germ-tubes were produced as well as what Corner (1935) has called tertiary growing-points (*h*). One germ-tube was normally in the ascendant and grew out

TEXT-FIGURE. All drawings in this figure have been made from living material with the aid of a camera lucida, using a Spencer Lens microscope and a $\frac{1}{8}$ -in. objective and $\times 10$ ocular. Drawings *a-k* are of *P. tritici*, *l-z* of *E. graminis*. The globules of cell contents at the broken ends of the leaf hyphae of *P. tritici* are marked by shading. Tertiary growing-points are shown growing from most spores of *E. graminis*.

The arrows show the points of penetration of the membrane. Points of contact with membrane are marked :

The membranes on which the specimens grew contained. *a-c*, collodion ICI 2644, paraffin wax 52° C.; *d, g, j*, collodion BDH (dated 1936), paraffin wax 36° C., 49° C.; *e, f, h, k*, collodion BDH (dated 1936), paraffin wax 52° C.-65/71° C.; *m, n, o*, collodion ICI 2644, paraffin wax 52° C.; *p, s*, collodion ICI 2644, paraffin wax 36° C.; *q, r, t*, collodion ICI 2644, paraffin wax 52° C.-65/71° C.; *u, v, w, x*, gelatin, paraffin wax 52° C.; *y, z*, collodion ICI 2644, paraffin wax 52° C.

a Leaf hyphae, unbranched and non-septate, with tapered apices. *b* Leaf hyphae, slightly branched and septate, with tapered apices. *c* Leaf hyphae, branched and much septate, with blunt apex. *d, g, j* Leaf hypha, septate, outgrowths with black spots, no penetration. *e, h* Leaf hypha, septate, outgrowths penetrating and swollen below membrane. *f, k* Leaf hypha, septate after contact with membrane, outgrowths penetrating and swollen below membrane. *l* Germ-tube growing in the air. *m* Germ-tube with three main branches, membrane soaked in 100 per cent. alcohol. *n* Germ-tube with finger-like processes, membrane soaked in 100 per cent. alcohol. *o* Germ-tube with numerous incipient growing-points, membrane soaked in 100 per cent. alcohol. *p* Two germ-tubes with club-shaped appressoria, developed after contact, showing black spots. *q* Germ-tube with curved appressorium and a black ring. *r* Germ-tube with double headed curved appressorium and two black rings. *s* Germ-tube with twisted stalk before contact, appressorium and black ring. *t* Germ-tube curved, septate appressorium and black ring. *u* Germ-tube with club-shaped appressorium. Penetration point visible as a small dot. Ball-like swelling between wax and gelatin. *v* Germ-tube with club-shaped appressorium, and a black spot. A tertiary growing-point has formed a swelling between wax and gelatin. Penetration point visible as a small dot. *w* Germ-tube with branches from lower part of septate appressorium. A bilobed swelling towards apex of appressorium, between wax and gelatin. Penetration point visible. *x* Reconstructed drawing of side view showing the relative position of appressorium above wax layer, fine penetration tube through wax, and swelling between wax and gelatin. *y* Germ-tube with curved appressorium and penetrating apex with a double growing-point. Side view to show relative position. Note large diameter at penetration. Membrane very thin. *z* Germ-tubes with appressoria and penetrating apices continuing growth as hyphae. Note large diameter at penetration. Membrane very thin.

as a hypha. Growth continued until the hypha was about 5–10 times the longer axis of the spore, one or more septa being usually visible. Shortly after growth ceased the contents of both spore and germ-tube appeared as if plasmolysed, and no recovery from this condition has been seen. The time taken to reach this stage was about 3–4 days. If contact was made with a membrane the form of growth varied with the type of membrane.

On thoroughly dried paraffin-wax (52° C.) collodion membranes, soaked in 100 per cent. alcohol, a branched hyphal growth was seen (*m*, *n*, *o*). The diameter of the germ-tubes was smaller than usual. At times two or only three of the finger-like branches were formed, and these were of some length (*m*). In other cases six or eight or more processes were produced on either side of the germ-tube (*n*, *o*); these looked rather like those developed from the main haustorial swelling in the host cell. There was also a suggestion of the repeated formation of daughter growing-points observed in rust germ-tubes (Dickinson, II, 1949).

On collodion membranes containing paraffin waxes with low congealing-points the germ-tubes developed a swelling towards the apex, which became a typical appressorium as on the host (*p*). The distance between the club-shaped appressorium and spore varied considerably (*p*, *s*). Sometimes an undulating growth was seen in the hypha (*s*), similar to that in *P. graminis* (Dickinson, II, 1949). On occasion these appressoria burst and their contents spread out as observed in rusts (Dickinson, II, 1949). The actual burst has not been seen, and it is not certain if there was any considerable swelling before the burst as in rusts (Dickinson, II, 1949).

With paraffin waxes of higher congealing-points in the membranes the appressorium became curved or sickle-shaped (*q*). In addition branches arose from segments of the septate hypha or the appressorium (*r*, *t*). Towards the tip of the appressorium a black spot, semicircular or in the form of a ring, was as a rule present (*p*). Usually the black spot originally semicircular became later ring-like (*q*, *r*, *s*). These spots have also been seen on the host plant. Whenever an attempt was made to move the appressorium by a glass rod, its apex was always found—if a black spot was present—to be securely fastened to the membrane. Movement with a glass rod generally caused the black spot to become ring-like or vice versa or caused its disappearance. As in rust germ-tubes (Dickinson, II, 1949), no sign of a mucilaginous condition of any part of the hyphal or appressorial wall was seen in spite of repeated tests with indian ink. The black spots are thought to be due to air pockets causing diffraction at or around the point of formation of the penetration tube. It is suggested that such air pockets arise during the early stages of penetration as a result of the appressoria and membranes becoming separated, owing to the force exerted by the potential penetration tube lifting the appressorium near the point of potential penetration. They have never been seen after penetration. The curved or sickle-shape of the appressorium (*g*), which developed later, is due, it is thought, to continued growth in length of the hypha or appressorium after its tip has become securely fixed. After

2 or 3 days the contents of these appressoria and hyphae appeared as if plasmolysed.

Penetration of these membranes by outgrowths from the appressoria was very rare, and no outgrowths have ever been seen from appressoria without penetration of the membrane. If, however, the membranes were made very thin, then penetration readily took place. The hyphae and appressoria grew through these thin membranes without any visible check to their growth, and with little or no change in diameter (y , z); their growth continued for a time in the liquid below the membrane. This penetration of thin membranes was quite distinct from that by outgrowths from the appressoria, now to be described.

In a few cases on collodion membranes containing paraffin wax, and commonly on double membranes (paraffin wax overlying gelatin), penetration by an outgrowth from a typical appressorium and from tertiary growing-points took place (u , v , w). The diameter of the penetrating outgrowth or tube was much less than that of the germ-tubes, &c. The penetration tube, after passing through the wax layer, formed at its tip a single or bilobed swelling of considerable size (u , w). The position of this swelling between the wax and gelatin was observed by staining the gelatin with a water-soluble dye. Careful focusing then enabled the surface of the gelatin to be traced and in all cases the swelling was between the wax and gelatin. A side view, made from drawings at different focal levels, showed the relative positions (x). No further development has been observed, though subsequent branching from parent appressorium or hypha has been seen. Owing to the difficulty of removing the gelatin part of the double membrane, the cytological preparations so far made have been unsatisfactory.

DISCUSSION

The nature of the structures formed on artificial membranes by hyphae arising from leaves infected with *P. triticina* and by germ-tubes of *E. graminis* must at present be decided by the conditions of their formation and by their morphology. These structures are a reaction to contact stimuli of some kind, for they were only formed after contact with a membrane. They were always associated with penetration. Incipient formation accompanied the early stages of penetration in *P. triticina*, but not in *E. graminis*. The limitation of the structures to a ball-like swelling below the membrane appeared characteristic. In this it was distinct from the penetration of thin membranes by *E. graminis* and that already described in germ-tubes of *P. triticina* (Dickinson, II, 1949). The limitation of growth of the structures is similar to that of the haustoria in the host. In *E. graminis* the difference in diameter between the hyphae penetrating thin membranes and the true penetration tube was marked. The diameter of the stalk in *P. triticina* was much larger than that of a haustorium in the host (see Table). In *E. graminis* the diameter of the penetration tube was similar whether formed on the membranes or on the plant. The length of the stalks of the structure was very short in both fungi; this might

be due to the thickness of the membranes used. The diameters of the swellings themselves were comparable to those of haustoria in the host, though those of *P. triticina* may develop a conical form in the leaf. In *E. graminis* on the host a number of finger-like processes are commonly, but not invariably, produced from the main body of the haustorium. These may bear some relation to those formed by its germ-tubes on alcohol-treated membranes.

TABLE

A Comparison in the Two Fungi of the Relative Size of the Haustorium as formed in the Leaf and on an Artificial Membrane

	<i>P. triticina.</i>		<i>E. graminis.</i>	
	Haustorium		Haustorium	
	In leaf.	On membrane.	In leaf.	On membrane.
Diameter of stalk	1 μ	3 μ	1-1.5 μ	About 1 μ
Length of stalk	> 20 μ	1-3 μ	> 15 μ	1-3 μ
Diameter of swelling	Up to 20 μ	10-20 μ	7-10 μ	10-20 μ

The nature of the stimulus producing these structures has not been demonstrated, but they were only formed after contact with a membrane. So far they have been observed in *P. triticina* on one type of membrane only, and this contained paraffin wax. In *E. graminis* they have been seen both on collodion and on gelatin membranes, but these membranes had a common constituent, paraffin wax. Hence it is possible that the stimulus resulting from contact with a membrane was chemical rather than physical in nature.

Thus contact with the host cell-wall or with a membrane was followed by penetration and then by growth which was limited to the formation of haustoria or the structures previously described. This similarity of behaviour after contact either with a cell-wall or with an artificial membrane was observed in both fungi. In addition there was a general agreement in size between haustoria and these structures excepting the length of the stalks, and in *P. triticina* their diameter. These observations suggest that the structures formed after contact and penetration of a membrane are haustoria.

In *P. triticina* the formation of both substomatal vesicles by the germ-tubes and of haustoria by hyphae from the leaf followed contact with a membrane. The differences between them were the absence of septa in the germ-tubes, of the red coloration, and of bursting in the hyphae from the leaf.

In *P. triticina* the number of nuclei present in the non-septate hyphae from the leaf were very few, while the number present in the septate hyphae were two or more in each cell. As septation was only observed in hyphae in contact with a membrane, it is probable that nuclear division also resulted from contact with a membrane; this nuclear behaviour is similar to that in the germ-tubes.

It would seem that in obligate parasites contact with the cell-wall provides

the stimulus for haustorial formation and, in rusts at least, for nuclear division and septation. This emphasizes the previous deduction (Dickinson, I, 1949) that growth under artificial conditions may occur only on particular surfaces. Further experiments should show if food absorption is limited to the haustoria themselves.

The similarity in rusts and in powdery mildews of the growth forms resulting from contact suggests that the haustorial mechanism of obligate parasitism is an example of convergent evolution in Ascomycetes and Basidiomycetes.

SUMMARY

The structures formed after contact and penetration of certain artificial membranes by the mycelium from leaves infected with *Puccinia triticina* and by the germ-tubes of *Erysiphe graminis* are described. The correspondence in method of formation and in size between these structures and the haustoria in the host is very close. It is concluded that these structures are haustoria formed on artificial membranes.

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Experimental and Analytical Studies of Pteridophytes

XV. Further Observations on the Effect of Different Concentrations of Oxygen on Meristems of Certain Ferns

BY

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With Plate XI and six Figures in the Text

INTRODUCTION

IN an earlier communication Wardlaw and Allsopp (1948) described the external morphology of the plantlings which developed on pieces of rhizome of *Matteuccia struthiopteris* and *Onoclea sensibilis* which had been maintained in atmospheres containing 6, 11, 21, and 45 per cent. of oxygen in nitrogen. The further growth in the several atmospheres of young plantlings which had begun their development in air, and of dormant and active apices of *Dryopteris aristata*, was also studied. No differences in external morphology resulted from growth in the different oxygen concentrations. The suggestion made by White (1939) that a diminished supply of oxygen may be a factor in the differentiation of organs thus received no support.

In the present paper an account is given (i) of the internal structure of the materials indicated above and (ii) of certain interesting abnormal developments which resulted from the growth of meristems of *Matteuccia* and *Onoclea* in pure oxygen. The first part of the investigation was carried out with the object of testing the hypothesis that oxygen concentration plays an important role in tissue differentiation, while the study of the abnormal growths described in the second part supplies further information as to the growth potentialities of the detached meristems of *Onoclea* and *Matteuccia* and may thus assist in the interpretation of the conditions governing their normal development.

MATERIALS AND METHODS

The plants of *Dryopteris aristata*, *Onoclea sensibilis*, and *Matteuccia struthiopteris* grown in the several different concentrations of oxygen were fixed in chromacetic solution and subsequently stained with safranin and Delafield's haematoxylin. The fixation and staining of the growths obtained in pure oxygen were rather unsatisfactory, possibly because of the presence of an investing periderm.

For the growth of bud rudiments in pure oxygen the apparatus used was that described by Wardlaw and Allsopp (1948). This experiment was begun somewhat late in the season (August 19), but in comparable materials, supplied

with 6 and 21 per cent. oxygen, growth proceeded normally, although rather more slowly than earlier in the year. The rhizome pieces of *Onoclea* and *Matteuccia* were prepared as in the earliest experiments and both clear and darkened tubes were used. On September 22 pieces of decapitated rhizome, which had been developing on peat in a damp greenhouse since the beginning of the experiment 34 days previously, were also placed in the growth-tubes: in *Onoclea* the bud rudiments or detached meristems had produced cylindrical outgrowths 0.4–0.7 cm. in length, while in *Matteuccia* only small green cushions had developed. On October 20 rhizome lengths of *Matteuccia* and *Onoclea* which had been maintained in pure oxygen, and had developed the abnormal growths described below, were transferred to clear tubes supplied with 21 per cent. oxygen. Growth was continued until December 18, when a final examination was made.

ANATOMICAL OBSERVATIONS ON PLANTLINGS IN OXYGEN-NITROGEN MIXTURES FROM THE OUTSET

An anatomical investigation of the outgrowths from *Matteuccia struthiopteris* and *Onoclea sensibilis* rhizomes and from apices of *Dryopteris aristata* showed that no structural differences had developed in the several materials as a result of exposure to the different concentrations of oxygen. In the *Onoclea* plantlings, both in light and dark, the characteristic development from a protostele through solenostely to a normal dictyostele and the characteristic organization of the apical meristem were consistently observed. Histologically the mature stems were virtually identical. The leaves were also closely similar, with epidermal cells of the same shape and size and no evident difference in stomatal number. The etiolated materials showed fewer differences from normal than is characteristic of spermatophytes. The most notable difference lay in the greater parenchymatous development found in the etiolated plantlings. In *Matteuccia* also no differences were noticed in plants from the different oxygen concentrations. The presence of detached meristems was observed in each case. The contrast between etiolated and light-grown plants was more marked than in *Onoclea*, but there were few histological differences.

In *Dryopteris* the apex showed the same manner of growth in each specimen and no structural differences were detected between light- and dark-grown plants.

EXTERNAL MORPHOLOGY OF GROWTHS DEVELOPED IN OXYGEN

(a) *In oxygen from the outset*

In marked contrast to the plantlings produced by *Onoclea* and *Matteuccia* in oxygen concentrations ranging from 6 to 45 per cent. are the strange growths which appear when pieces of rhizomes are exposed to a stream of undiluted oxygen. Although clearly abnormal, these growths afford further information of the potentialities for development of the detached meristems of these fern rhizomes.

After 34 days' exposure to pure oxygen some of the detached meristems had succumbed to the abnormal conditions but others had retained their capacity for growth. In *Onoclea* they had grown out as rounded masses of tissue with no indication of organ formation, while in *Matteuccia* only a slight outgrowth was evident. After 62 days' exposure in all, the *Onoclea* growths had shown little further development and the *Matteuccia* rhizomes had produced similar tissue masses. In both plants similar growths were obtained in light and dark. The *Onoclea* growths were typically sub-spherical and had almost attained their maximum size after 62 days. After 121 days (December 18) they had shown little further growth but the surface had become dark and corky-looking. Text-fig. 1 illustrates a typical sample removed on this date. In *Matteuccia* the growth was always more irregular and slightly larger (Text-fig. 5). As in *Onoclea* the surface was dark and cork-like. By contrast, comparable rhizome pieces grown in 6 and 21 per cent. oxygen during the first 62 days of the experiment had shown the normal type of development.

(b) *In oxygen after initial growth in air*

Pieces of rhizome placed in the growth-tubes after 34 days' initial development in air were somewhat different in behaviour. At the time of introduction some growth had already occurred and the *Onoclea* rhizomes bore small cylindrical plantlings 0.4–0.7 cm. in length, while on the *Matteuccia* rhizomes only small green cushions had appeared. In 6 per cent. oxygen the subsequent growth of the plantlings was more rapid than that of the plantlings which had grown in this atmosphere from the outset, thus confirming the results of earlier work (Wardlaw and Allsopp, 1948).

After 28 days in oxygen the detached meristems of *Matteuccia* had shown little further growth. The small outgrowths originally present on the *Onoclea* rhizomes had formed several juvenile leaves, but already these showed signs of tissue necrosis. After 87 days the *Matteuccia* rhizomes had developed irregular growths rather larger than those found on the rhizomes maintained in oxygen from the outset. On the *Onoclea* rhizomes all plantling tissue had suffered necrosis while the basal tissue developed from the detached meristem had undergone considerable proliferation (Text-fig. 4). In some instances tissue masses had developed from meristems which had shown no external indication of activity at the time of transfer to oxygen.

(c) *Further growth after transfer from oxygen to air*

When pieces of rhizome which had been held for 62 days in pure oxygen were transferred to 21 per cent. oxygen, the dark corky-looking outgrowths gave little promise of further development, but after 59 days numerous bright-green outgrowths had emerged through the dark surface layers. In *Onoclea* (Text-figs. 2, 3) the smaller outgrowths were as yet externally undifferentiated but the larger ones consisted of apical growing regions with one or more young leaves, while in *Matteuccia* one plantling had produced a young root of

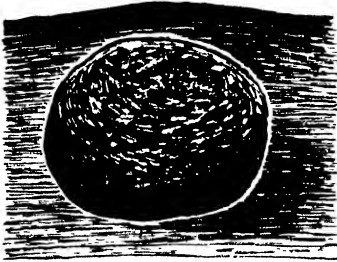


FIG. 1

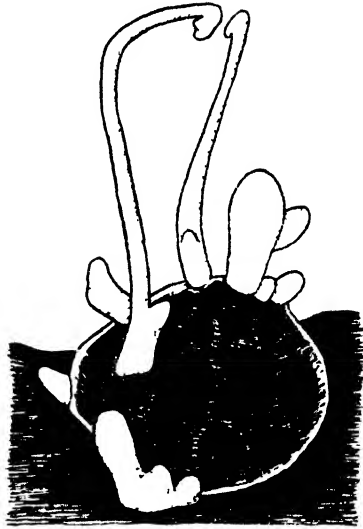


FIG. 2

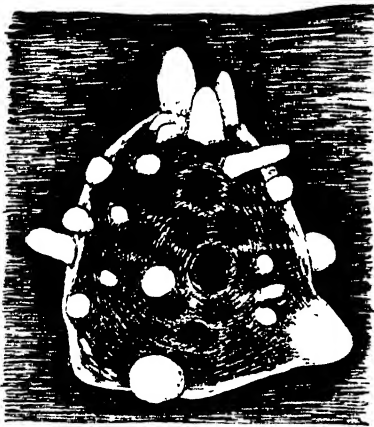


FIG. 3

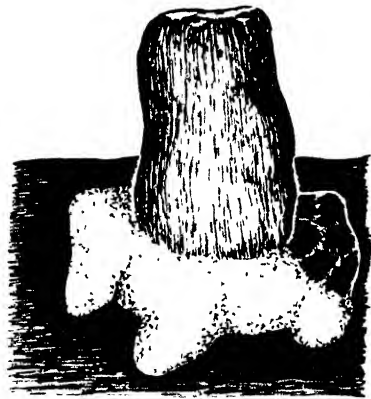


FIG. 4

TEXT-FIGS. 1-4 ($\times 10$). *Onoclea sensibilis*. Fig. 1. Growth developed from detached meristem after 121 days in undiluted oxygen. Figs. 2 and 3. Growths developed after 62 days in air following 59 days in oxygen. Fig. 4. Proliferation from base of decayed plantling after 87 days in oxygen following 34 days in air.

appreciable length (Text-fig. 6). These growths were much more attenuated and more numerous than those developed on normal rhizomes, where one plantling to a detached meristem is the most frequent condition. The disrupted appearance of the surface gave evidence of a general swelling of the *Onoclea* growths; but this was not clearly shown on the more irregular surface of those of *Matteuccia*.

ANATOMICAL OBSERVATIONS ON GROWTHS DEVELOPED IN OXYGEN

(a) *In oxygen from the outset*

Onoclea. A median longitudinal section through the growth on an *Onoclea* rhizome, exposed to undiluted oxygen for a period of 34 days, is illustrated in Pl. XI, Fig. 1. Serial sections show that the structure is very regular and apparently the product of growth over the entire surface. The outermost tissue consists of a periderm of several layers resembling that of

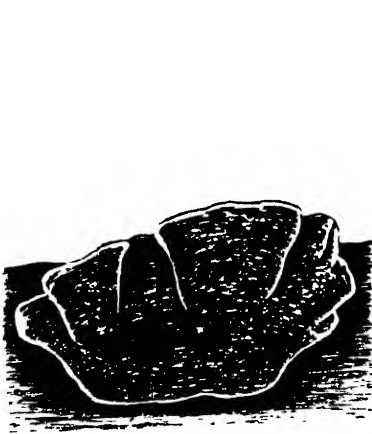


FIG. 5

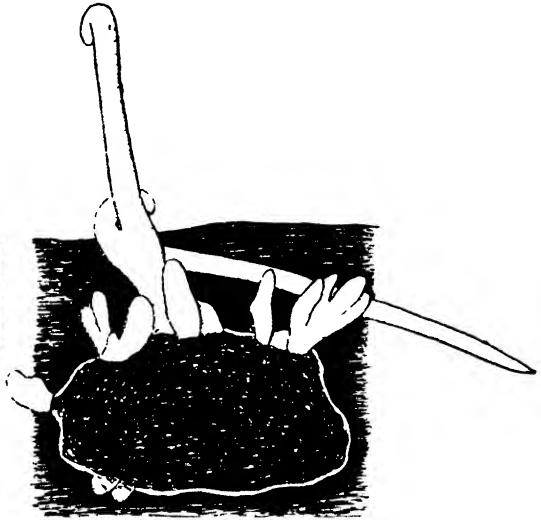


FIG. 6

TEXT-FIGS. 5-6 ($\times 10$) *Matteuccia struthiopteris*. Fig. 5. Growth developed from detached meristem after 121 days in undiluted oxygen. Fig. 6. Growth after 62 days in air following 59 days in oxygen.

many higher plants, the cells being in regular radial rows continuous with the meristematic cells within (Pl. XI, Fig. 2). In many cases the cells have a characteristic internal marking, possibly the remains of the original protoplasmic contents, while the cell-walls are usually brown but not appreciably thickened. Within the periderm is a meristematic region of several layers of deeply staining cells. This meristematic tissue usually extends over most of the sub-spherical outgrowth but is more active in the distal region. In the figured section (Pl. XI, Fig. 1) it appears as a dark region extending over approximately three-fifths of the outgrowth. Within this region in the upper part of the growth are feebly staining cells with prominent nuclei, which gradually pass into the large central mass of tracheides intermixed with elongated cells with dense granular contents. The tracheides extend a little way into the parent rhizome, but there is no connexion with the meristemes of the latter.

Matteuccia. The growths developed on the *Matteuccia* rhizomes are of

more irregular structure. A periderm similar to that observed in *Onoclea*, and frequently with the same characteristic markings in the cells, completely invests the outgrowth. Growth apparently occurs over the whole surface but with localized intensification of activity. Within the deeply staining meristematic region there is a transition to elongated cells of what appears to be an incipient vascular region. In most cases there was no connexion with the steles of the parent rhizome, but in one instance the incipient vascular tissue of the outgrowth and the parent steles were conjoined by tracheides. In this particular instance tracheides and an irregular endodermis were associated with two regions of active growth, but disappeared basipetally into the general mass of the growth. The active regions approached the structure of an apical growing-point, but there was no distinct apical cell, and cell divisions were less regular than in a normal apex.

(b) *In oxygen after initial growth in air*

The rhizome pieces maintained in air for 34 days before transfer to the oxygen tubes showed certain differences from those grown in oxygen from the outset. It has already been mentioned that in *Onoclea* small plantlings with leaves developed, but speedily became necrosed, while the basal tissues underwent further proliferation. Other meristems previously dormant produced rounded bulges, similar in external appearance and internal anatomy to those already described for rhizomes developing in undiluted oxygen. In *Matteuccia* plantlings were never formed, but irregular outgrowths resulted which were rather larger than those produced on rhizomes maintained in oxygen throughout.

A median longitudinal section of one of these growths of *Matteuccia* is illustrated in Pl. XI, Fig. 3. Pl. XI, Fig. 4, is a more highly magnified portion of the outer tissues from an adjoining section, while Fig. 5 is from the central tissues of the section shown in Fig. 3. It would seem that there has been growth over the entire surface. In this example periderm formation has not extended to the production of many layers. Cells in process of conversion to periderm are seen in the outermost layers of Fig. 4. Beneath the periderm is a region of meristematic cells with deeply staining contents which extend almost completely round the periphery of the growth. Within are several layers of parenchymatous cells with fairly regular radial arrangement. At approximately one-fifth of the distance between the periphery and the centre of the growth is an irregular endodermis almost completely enclosing the central tissues. A zone of fairly deeply staining parenchymatous cells with dense contents passes into a region containing short tracheides (Pl. XI, Fig. 4) and finally into the central axis of the growth, which consists of elongated cells intermixed with occasional spiral tracheides. Serial sections show that at several points there has been an approach to the formation of apical growing-points. This is indicated by the presence of local protrusions surrounded by characteristic scales. In each case the local development is associated with the presence of tracheides and an endodermis which pass back to join the central mass. The larger outer cells of the projections have lost their contents and

are of corky appearance, while the cells below have undergone repeated divisions to produce regular radial files of cells.

(c) *Further growth after transfer from oxygen to air*

The growths transferred to 21 per cent. oxygen after a previous exposure to pure oxygen have also shown features of interest. In sections of the *Onoclea* material illustrated in Text-figs. 2 and 3 and of the *Matteuccia* material (Text-fig. 6), the main mass of the outgrowths was not appreciably different in structure from the growths maintained in oxygen throughout. In every case the secondary growths projecting from the parent growths had arisen endogenously from the deeply staining meristematic cells within the periderm. The origin of a new growth in *Matteuccia* is illustrated by Pl. XI, Fig. 6; the new growth, with remains of the periderm adhering to the upper side, is seen pushing out on the right of the original growth. These young growths had no distinctive apical cells, but in the larger structures shown in Text-figs. 2 and 6 normal apical regions had been differentiated. This was found in both *Onoclea* and *Matteuccia*. The young plantlings contained small vascular cylinders with tracheides and endodermis, but these ended blindly in the tissues of the parent growth. In contrast with these vascular developments the *Onoclea* growth illustrated in Text-fig. 2 and that of *Matteuccia* in Text-fig. 6 were without tracheides in their central region, this consisting solely of elongated cells. Small tracheides were present at the base of the secondary growth shown in Pl. XI, Fig. 6, but these are not shown in the section illustrated.

DISCUSSION

An anatomical examination of plantlings of *Matteuccia* and *Onoclea* and of shoot apices of *Dryopteris* has revealed no notable differences between those grown in 6, 11, 21, and 45 per cent. oxygen. This finding is in conformity with the earlier observations of Wardlaw and Allsopp (1948) that the external morphology of these plants was closely comparable in the different oxygen concentrations. The chief difference observed was in the rate of growth, not in the final product. These experimental data thus provide no support for the suggestion of White (1939) that a limited supply of oxygen may be one of the factors responsible for the differentiation of organs and tissues. This suggestion was based on the observation that undifferentiated cultures of a *Nicotiana* callus produced leafy shoots when immersed under 8 mm. of a liquid culture medium. It now seems probable that factors other than, or in addition to, low oxygen concentration were involved in the differentiation found in White's tissue cultures. A greater opportunity for the diffusion of hormones or other soluble substances into the liquid medium might, for example, be suggested as a possible factor. Levine (1947) obtained complete differentiation of stem, root, and leaves in carrot meristem cultures without any indication that altered oxygen gradients were involved.

In the normal growth of pieces of *Onoclea* and *Matteuccia* rhizomes, as described in detail by Wardlaw (1943, 1943a, 1945), one or more apical cells

arise in the surface layer of the cushions of green tissues (developed from the detached meristems) and each gives rise to a typical plantling. A different behaviour was observed when portions of the rhizomes of these ferns were maintained in undiluted oxygen, abnormal cushion-like structures with dead superficial cells and a characteristic periderm being formed. In the absence of plantling formation these cushions underwent considerable development and fairly large structures were eventually formed. The continued growth of the cushions was probably due to the absence of inhibition by the plantlings. This view is supported by the results of introducing into the undiluted oxygen rhizome pieces of *Onoclea* bearing small young plantlings: the plantlings became necrosed, but the tissues of the basal cushion often underwent further proliferation and yielded fairly large tissue masses. In most cases, prior to necrosis, the plantlings continued their development for a time producing several leaves which soon suffered a speedy disintegration. These leaves were present only as minute primordia when the rhizomes were placed in oxygen. This observation indicates that once an organ is initiated its development may continue for a time, even when external conditions are unfavourable for its continued existence.

The structure of the anomalous outgrowths varied to some extent with their size and activity. In all cases the characteristic periderm was produced. A similar periderm has already been reported by Holden (1912, 1916) as a wound reaction in certain filicinean petioles. It is a feature of some interest that the cells within the periderm retain their totipotency and when subsequently transferred to air give rise to plantlings somewhat attenuated but otherwise of normal structure. A similar production of numerous but attenuated plantlings from the basal cushion was observed by Wardlaw (1943a) in instances where the original plantlings had been mechanically injured or damaged by desiccation.

In the large cushion-like growths of *Onoclea* a considerable central mass consisting of tracheides intermingled with other elongated cells was present. In the less vigorous growths the central tissue consisted of elongated cells without tracheides. The outgrowths of *Matteuccia* were closely comparable. The most elaborate growths were suggestive of an original protostelic shoot which had suffered a degeneration of the apical meristem and an expansion of the central tissue. The arrest of apical development followed by more general surface growth in a young plantling similar to that illustrated by Wardlaw (1946, Text-fig. 6) would yield a like result.

The vascular tissues of the growths developing on transfer from oxygen to air are of considerable interest. The intense local meristematic activity of these secondary growths is always associated with the formation of tracheides, and as differentiation of a normal apex proceeds a definite vascular system delimited by an endodermis is developed. The vascular system of the secondary growths ends blindly in the parenchyma of the parent cushion. These several observations have a bearing on hypotheses advanced to explain the initial differentiation of vascular tissues. Suggested factors were grouped

by Lang (1915) in three categories: (1) functional stimuli, (2) the inductive influence of older preformed parts on the developing region, and (3) formative stimuli of unknown nature proceeding from the developing region. These possibilities have been discussed subsequently by Wardlaw (1944) with special reference to the data available for Pteridophytes, the third group receiving special attention. He pointed out that in the region of active synthesis at the apical meristem 'enzymes and other substances, e.g. auxins, possessing important physiological properties are known to result. These will tend to diffuse or otherwise move from the region of highest concentration—the meristematic cells in which they are produced—into the adjacent cells, and these, as also those at a distance, are therefore liable to undergo changes in their physiological and structural properties.' In the same paper evidence was presented of a close relationship between the presence of an active apical meristem and the initial differentiation of vascular tissue, and of an absence of such differentiation accompanying the disappearance of an apical meristem. From these and other considerations the following working hypothesis was advanced: 'Wherever the apical meristem of a shoot, bud, leaf or root is in a state of active growth of such a nature that the distinctive character of the meristematic cells is maintained, the initial differentiation of vascular tissue will be observable immediately below the apex and in the path of substances diffusing from it, one or more of these substances being causally involved in that process.' A similar hypothesis has been advanced independently by Camus (1944) to explain the production of vascular tissue subjacent to developing buds on excised fragments of endive root cultivated *in vitro*.

The present work affords further evidence of the differentiation of vascular tissue in relation to meristematic activity, although the presence of a normal apical meristem would not appear to be necessary. Thus the general peripheral meristem of the anomalous growths developed in pure oxygen is associated with a fairly symmetrical core of vascular tissue, whereas the secondary growths arising on subsequent transfer to air develop their own vascular supply, which remains unconnected with the original vascular core. This latter observation is opposed to the view that vascular differentiation is induced by stimuli from the older preformed vascular regions.

Although the dependence of the initiation of vascular tissue on meristematic activity seems convincingly demonstrated, the hypothesis that this is the consequence of diffusion of substances from the apex requires further support, for as pointed out by Wardlaw, the relevant observations may be accounted for by the alternative hypothesis of abstraction of substances by the cells of the apical meristem from the adjacent underlying tissue. The results reported in the present paper do not assist in the clarification of this issue. Either of the hypotheses would account for the observation that development of vascular tissues occurs in regions where the disposition and degree of activity of the overlying meristematic cells would lead one to expect maximal diffusion gradients. In addition, the bulk of the vascular elements are elongated in the direction of greatest movement, a circumstance which suggests

that diffusion gradients may play a role in the orientation of vascular tissues. A similar view was advanced by Simon (1908) who observed, in certain angiosperms, that new vascular connexions start from the basal ends of severed strands and grow from these towards upper severed ends, or, if these are not within reach, to other unsevered bundles. A directive influence is evidently at work and Simon suggested that a gradient of water deficit is responsible, the new vascular tissue first appearing in tissues relatively depleted of water. A gradient in the stimulating substance postulated by Wardlaw would, of course, have a similar effect. The importance of gradients in differentiation and development has been discussed extensively by Child (1941), but principally with reference to animal data. Apart from the investigations of Prat (1945) and Van Fleet (1948) the study of gradients in plant tissues has received little attention. In view of the directive influences apparently at work in the differentiation of vascular elements, however, it would appear that a more extensive knowledge of the physico-chemical and physiological gradients existing in plant tissues, especially in the apical growing-points, might throw further light on the factors underlying the differentiation of vascular and other plant tissues.

SUMMARY

Anatomical observations have revealed no differences in the structure of growing shoot apices of *Dryopteris aristata* and of plantlings developing from detached meristems of rhizomes of *Onoclea sensibilis* and *Matteuccia struthiopteris*, when maintained in gas mixtures containing 6, 11, 21, and 45 per cent. oxygen in nitrogen.

In undiluted oxygen the rhizome lengths of *Matteuccia* and *Onoclea* developed abnormal periderm-covered, cushion-like growths from the detached meristems. When these structures were transferred to air, attenuated but otherwise normal plantlings arose endogenously from meristematic tissue which persisted within the periderm. The morphology and anatomy of these growths are described.

The experimental and anatomical observations are discussed with reference to hypotheses relating to the origin of tissue differentiation and, in particular, of vascular tissue.

The writer wishes to acknowledge his indebtedness to Professor C. W. Wardlaw for helpful advice during the investigation and in the preparation of this account, and to Mr. E. Ashby for the photographic illustrations.

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DESCRIPTION OF PLATE XI

Illustrating A. Allsopp's article 'Experimental and Analytical Studies of Pteridophytes. XV. Further Observations of the Effect of Different Concentrations of Oxygen on Meristems of Certain Ferns'.

Figs 1-2. *Onoclea sensibilis*.

Fig. 1. Longitudinal section through growth developed from detached meristem after 34 days in undiluted oxygen. ($\times 20$.)

Fig. 2. Periderm from a section of growth illustrated in Fig. 1. ($\times 200$.)

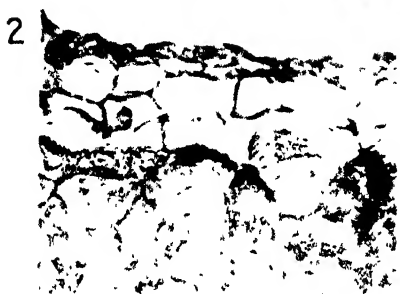
Figs. 3-6. *Matteuccia struthiopteris*.

Fig. 3. Longitudinal section of growth developed from detached meristem after 87 days in undiluted oxygen following 34 days in air. ($\times 20$.)

Fig. 4. Outer layers of a longitudinal section of growth illustrated in Fig. 3. ($\times 100$.)

Fig. 5. Central tissue of section shown in Fig. 3, showing tracheides. ($\times 200$.)

Fig. 6. Longitudinal section of growth from detached meristem after 62 days in air following 59 in oxygen. ($\times 20$.)



The Durian Theory or the Origin of the Modern Tree

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With thirty-six Figures in the Text

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PREFACE

THE value of a theory, as of any organization, is proved by what cannot be done without it. The theory which I offer seems useful because it reveals many insights into the nature of flowering plants, birds, and mammals—the life, that is, *par excellence*, of the tropical forest. It has led me to compare not only the forms of fruits and those of trees, but to think, at the same time, of tapirs, cycads, and brussels sprouts, of colours and monkeys, of fishes' eyes and modern patterns. It has led me to study the chalaza of the ovule as the neuropore of the gastrula, the formation of peltate scales, the lengths of funicles, and the weights of seeds, and to consider, beside the more obvious things, the biological significance of dangling, the origin of poppies, the disappearance of apes and elephants, the clamour of parrots, and that gap in palaeobotany—the beginning of flowering plants.

Hitherto the lead in evolutionary thought has been taken chiefly by zoology. This theory will turn attention, I hope, to tropical trees and, as a proof of the concept of Xerophyton, in a way not visualized by its author, will reawaken interest in that neglected work 'Thalassiophyta' (Church, 1919). There is now in the rain-forests of the equatorial belt a Xerophyton, representing a culmination of plant-evolution still in dynamic equilibrium with its degeneration-products, as the world ages, though the Thalassiophyton has gone for

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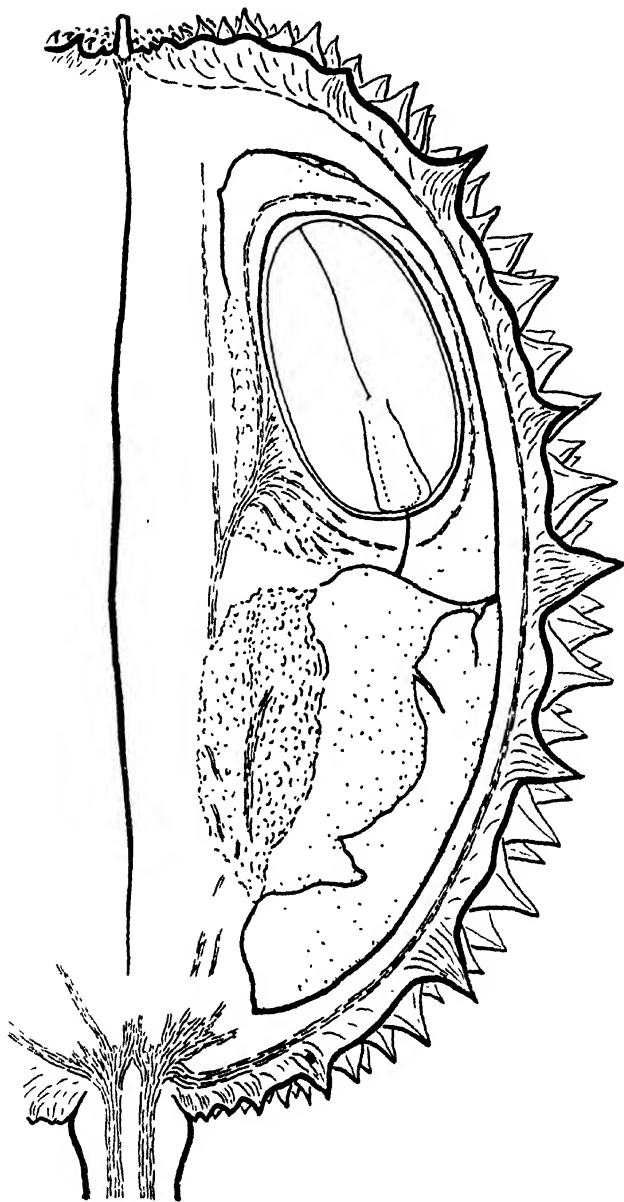


FIG. 1 The ripe fruit of *Durio zibethinus* about to open, in longitudinal section, showing the aril (dotted) round the seeds, the mealy placenta, and the vascular supply to the seed, aril, and spines ($\times 1$).

ever. Unluckily for my present purpose, the life of the tropical rain-forest cannot be compressed on to paper any more than it can be grasped by a foreign expedition. The subject is so vast and the objects so unfamiliar that, at best, I can hope to spur the younger generation to strive after the means of living in the tropics if it would consider evolution. That the seeming oddities of which I have written form a demonstrative whole should prove themselves that, without tropical orientation, biology is lost.

One Sunday in July 1944, when Professor Kwan Koriba was acting director of the Singapore Botanical Garden, we found in a patch of virgin forest on the island the fallen fruits of *Elaeocarpus javanicus* (Tiliaceae). They appeared to us to belong to the Meliaceae, Sapindaceae, Flacourtiaceae, Sterculiaceae, Bombacaceae, and even Connaraceae, until we could correct ourselves in the herbarium, but this very confusion led me to inquire. It seemed that this kind of fruit—a red loculicidal capsule with large black seeds hanging on persistent funicles and enveloped by a red aril (Fig. 6) must have been the ancestral fruit of this group of families. And if of this group, why not of all flowering plants?

I began work on this theory in Singapore during the last year of the war, and I express my gratitude to Professor Koriba for the part which he has played as protector of the scientific research of the Singapore Botanical Garden, as a critical adviser, and, if I may say so, my first convert to durianology.

WHAT IS THE DURIAN?

The durian (*Durio zibethinus*, Bombacaceae) is a lofty forest-tree of the Malayan region, now widely cultivated from India to New Guinea. It has rather small, simple leaves, slender twigs, and bunches of massive pink or white flowers, borne on the branches and giving place to huge, 5-shouldered, spiny, loculidial capsules which ripen olive to golden-yellow. In each cavity of the fruit are 1–5 large, light-brown seeds covered by the thick, creamy, white or yellow aril. The fruits do not open till they are fully ripe and have crashed to the ground. They then have a powerful and disgusting smell, of garlic and skatol, but the creamy aril is so delicious that the durian is the most popular and famous fruit of the East. Unripe fruits are heavily armoured with stout pyramidal spines, which are driven into the skin by the weight of the fruit when held in the hand, and they can rarely be attacked by animals, not even by squirrels, though the immature and mature seeds are highly nutritious and palatable.

Until recently, durian-trees were not selected. Wild trees in Malaya have as good fruits as those in cultivation which, indeed, is often little other than the protection of sporadic seedlings. In the forest they commonly occur in groves. In the season the smell of the fruits attracts the elephants which congregate for first choice; then come the tigers, pigs, deer, tapir, rhinoceros, monkeys, squirrels, and so on down to ants and beetles which scour the last refuse. The jungle-folk build tree shelters whence they can reach the ground

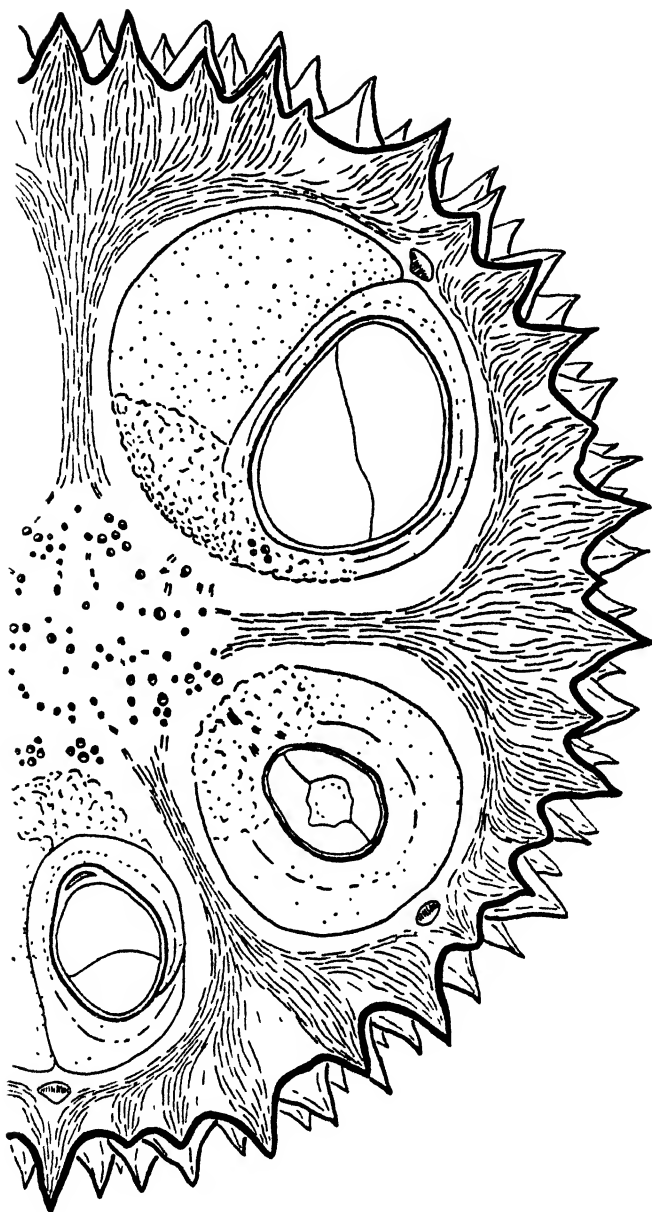


FIG. 2 The ripe fruit of *Durio zibethinus* in transverse section, showing the ails (dotted), the mealy placentas, and the vascular supply (consisting of numerous small axial bundles from which pass the bundles to the fruit-wall and spines, and a main longitudinal bundle on the outside of each loculus) ($\times 1$)

when a fruit drops, and whither they can climb again to safety. Under the big trees are leaning saplings, frayed bark, trampled shrubs, and churned ground, as scenes of elephantine supremacy.

The spines develop only under the initial peltate scales of the ovary, each

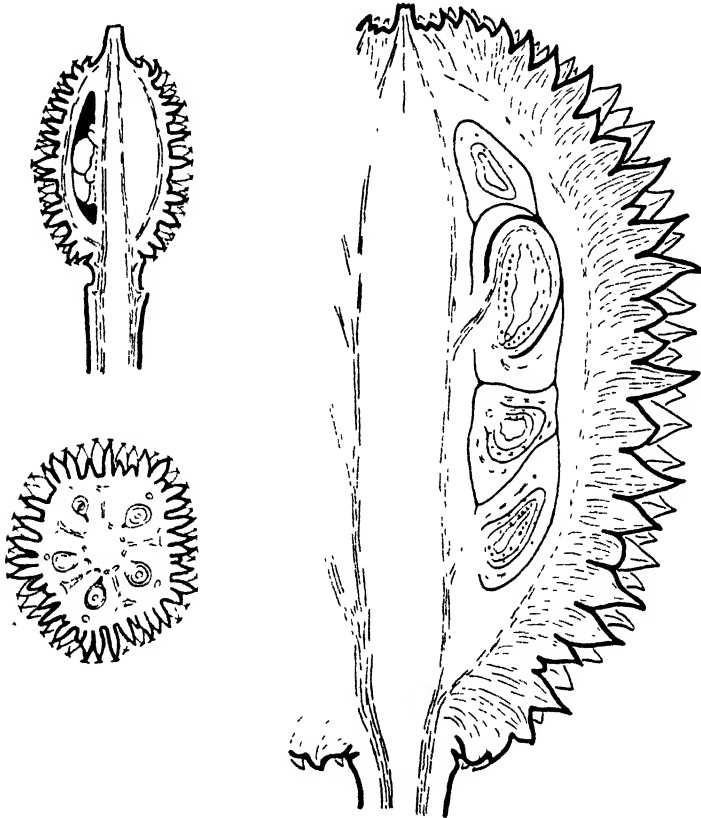


FIG. 3. Developing fruit of *Durio zibethinus*, shortly after pollination (in l.s. and t.s.), and when half-grown, to show the rapid growth of the aril, the development of the spines below peltate scales, and the vascular supply ($\times 1$).

spine bearing, as it were, a primary scale at its tip and secondary peltate scales on the sides.

The aril, as usual, develops only after pollination, but unfertilized ovules may develop the aril as the fruit sets.

There are about fifteen species of *Durio*, distributed through Siam, Burma, Philippine Islands, Malaya, Sumatra, Borneo, and Java. Most have incomplete arils or none at all, and some are cauliflorous rather than ramiflorous.

One species, *D. Griffithii*, has small, red fruits, softening when ripe, and black seeds with short red arils. The fruits are axillary on the leafy twigs and open on the tree so that the black seeds hang on the edges of the star-shaped fruit, as with *Sterculia* and *Sloanea*.

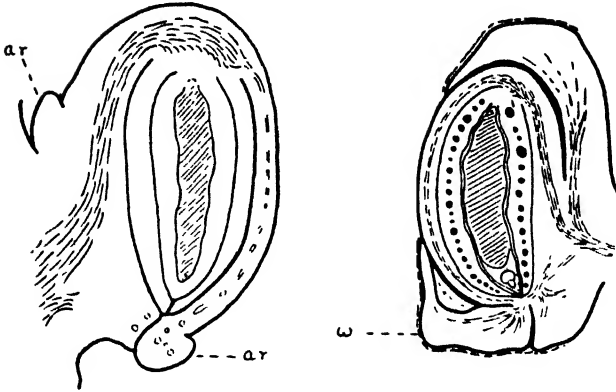


FIG. 4. Developing seeds of *Durio zibethinus*, in the same stages as in Fig. 3; the nucellus containing the embryo-sac fluid, the inner integument developing mucilage sacs (right figure, as black circles), $\times 15$ (left); $\times 2$ (right). *ar*, aril; *w*, waxy secretion of aril.

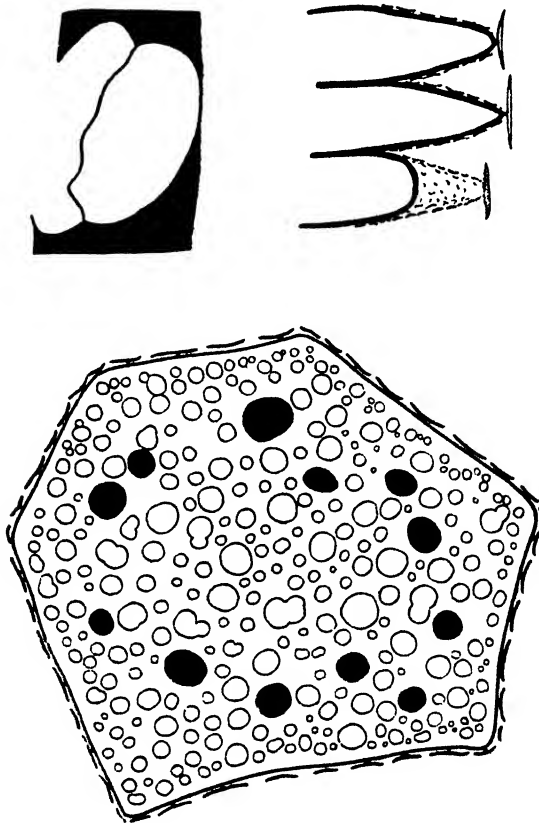


FIG. 5. *Durio zibethinus*, a section of the young fruit (above) with incipient aril and spines ($\times 7$); and a transverse section of a spine from a half-grown fruit, to show its complex vascular supply (the outer smaller bundles being only fibrous), the mucilage canals (black), and the peltate scales ($\times 15$).

Three other genera of the Bombacaceae have arillate seeds, namely, *Coelostegia* (Malaya, 2 spp.), *Neesia* (Malaysia, 10 spp.), and *Cullenia* (Ceylon, 1 sp.).

Only these four genera, all of south-east Asia, in the vast Bombacaceae-Malvaceae of several thousand species, have this type of capsular arillate fruit.

Problem. What is the origin of this huge armoured capsule, so fiercely sought after by wild animals, yet so rare as to occur in a mere sprinkling of tropical trees of this large series? It is, at once, a biological success and a curiosity. Why do durians exist?

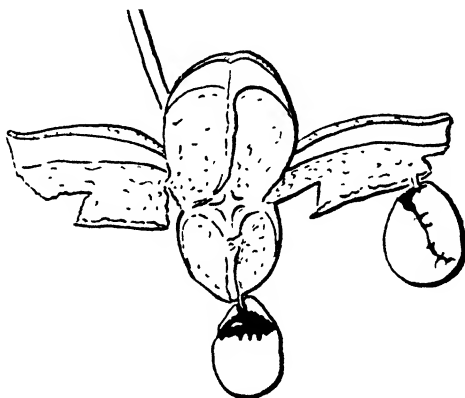


FIG. 6. Dehiscent fruit of *Sloanea javanica* (Tiliaceae-Elaeocarpaceae), showing the black seeds with red arils ($\times \frac{1}{2}$).

ARILLATE FAMILIES

The following are the main, if not all, families of flowering plants with arillate seeds.

A. All genera and species arillate

Myristicaceae: Stachyuraceae (1 gen., 2 spp., Japan, Himalayas).

B. Most genera arillate

Dilleniaceae, Connaraceae, Passifloraceae, Scitamineae (Musaceae, Marantaceae, Zingiberaceae).

C. Many genera arillate

Meliaceae, Celastraceae, Sapindaceae, Flacourtiaceae, Melianthaceae, Guttiferae (Clusiaceae).

D. Few genera arillate (number of genera in brackets)

Nymphaeaceae (2), Annonaceae (3), Monimiaceae (1), Berberidaceae (2), Papaveraceae (1), Linaceae, Malvaceae-Bombacaceae (4), Sterculiaceae (3), Tiliaceae (Elaeocarpaceae, 1), Leguminosae (Mimos. 2, Caesalp. 14, Papil. 1, Swartz. 1), Theaceae (1), Samydaceae, Rhamnaceae (1), Rhizophoraceae (3), Melastomaceae (4), Aizoaceae, Lecythidaceae (2), Thymelaeaceae (2), Apocynaceae (2), Commelinaceae (2).

E. Rudimentary aril

Ranunculaceae (Paeonia), Fumariaceae, Polygalaceae, Violaceae, Oxalidaceae, Bixaceae, Turneraceae, Tremandraceae, Euphorbiaceae, Leguminosae (Papilionaceae), Cactaceae, Liliaceae (2).

Only some forty-five families have arils, more or less. Only one large family is wholly arillate: six are mainly arillate. All these families are chiefly,

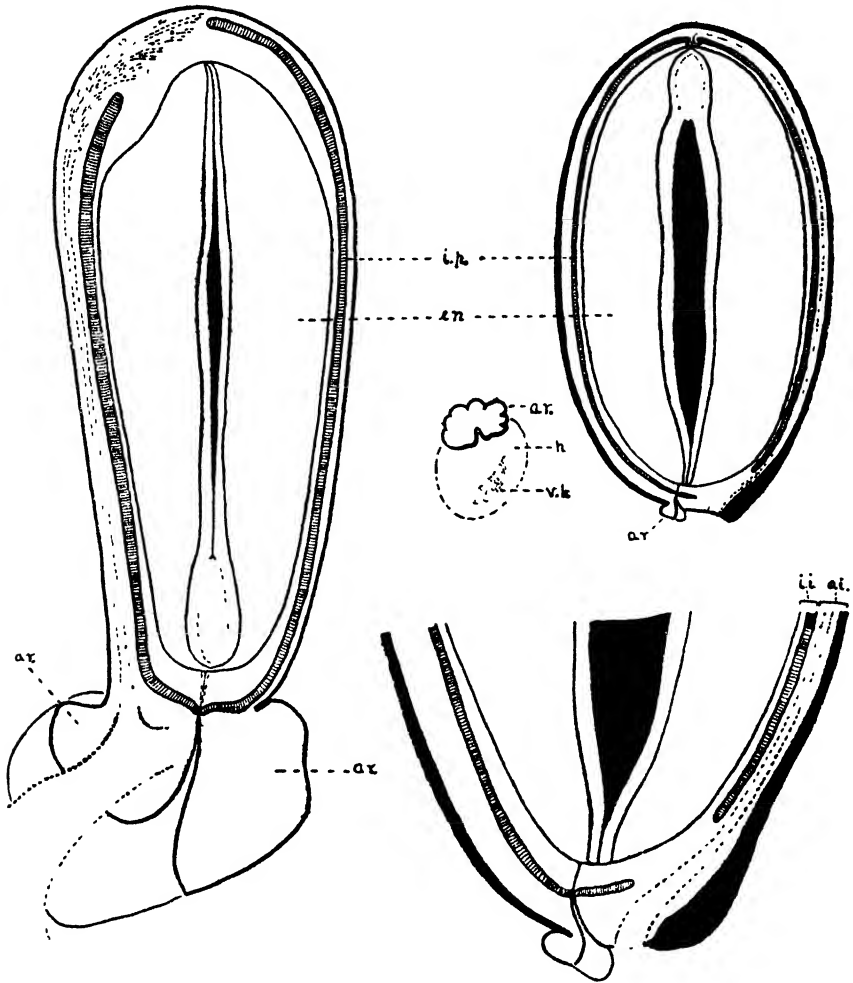


FIG. 7. Longitudinal sections of mature seeds of *Coelostegia Griffithiana* (left, Bombacaceae $\times 3$), and *Sterculia macrophylla* (right, the upper figure $\times 3$, the lower $\times 7$, the hilum $\times 7$). The seed of *Sterculia* has the embryo inverted, with radicle pointing to the chalaza: when the *o.i.* weathers off, the chalazal pore in the palisade of the *i.i.* appears as a false (but effective) micropyle. *ar.*, the aril (much reduced and limited to the micropyle and funicle in *Coelostegia*, entirely rudimentary in *Sterculia*, though bright yellow); *en*, endosperm; *h*, hilum; *i.i.*, inner integument; *i.p.*, the palisade of the inner integument; *o.i.*, the outer integument; *v.b.*, vascular bundle (dotted) of the hilum.

if not entirely, tropical. Most of the arillate seeds belong to tropical trees or woody climbers. Extremely few arils of any size occur in small plants, e.g. *Acrotrema* (Dilleniaceae).

Generic examples: *Myristica*, *Xylopia* (Annonaceae), *Wormia* (Dilleniaceae), *Connarus*, *Dysoxylon* (Meliaceae), *Leptonychia* (Sterculiaceae), *Guioa*, *Nephelium*,

Paulinia (Sapindaceae), *Tabernaemontana* (Apocynaceae), *Sloanea* (Tiliaceae), *Ravenala* (Musaceae).

Sterculia provides an example of a rudimentary aril, for several species have a minute yellow arillate cushion 1–2 mm. wide on one side of the micropyle.

Spines. As with the Bombacaceous genera, so in the other cases, these arillate capsules are often spiny (see p. 397).

Problems. Exactly as with *Durio*:

- A. Why are these successful fruits, much eaten by birds, bats, and arboreal mammals, so comparatively rare, even in secondary jungle where animal-distributed plants are common?



FIG. 8 The epigynous fruit of *Siparuna* sp. (Monimiaceae), irregularly dehiscent, the light-grey tuberculate seeds with dark-red aril (cf. the irregularly dehiscent fruits of some species of *Ficus*). A, the full-grown purple-red indehiscent fruit ($\times 1$); B, the dehiscent fruit with pink, mealy-granulate flesh in which the seeds are embedded ($\times 1$); C, a longitudinal section of the full-grown fruit showing the pulpy, connate carpel-walls ($\times 1\frac{1}{2}$); 3 seeds ($\times 2$). *a*, aril; *m*, micropyle; *r*, vascular bundle of the raphe; *t*, the testa.

- B. Why are there, in many genera, species showing all degrees of development of the aril from none at all to the complete aril, e.g. *Sloanea*, *Xylopia*, *Acacia*, *Dysoxylon*, as with *Durio*?
- C. Why are there, in related genera, so many transitions from this arillate capsule to dry capsules with dry, often winged, seeds (Meliaceae, Apocynaceae), to drupes (Annonaceae) or berries (Dilleniaceae) or nuts (Lecythidaceae)? Even in the one genus *Xylopia* there are arillate follicles, indehiscent berry-like follicles, and 1-seeded drupe-like follicles; and there is almost the same transition in *Pithecellobium* (Mimosaceae).
- D. Are these arillate fruits new and parallel inventions in these different families and genera? Or are they relics showing the ancestral conditions from which modern fruits such as the dry capsules, follicles, nuts, berries, drupes, and so on, have been evolved?

One or other of these two points of view must be correct.

ARGUMENT

- A. If these fruits are upgrade and recent, then:

a. Why should all these widely different families, e.g. Apocynaceae and Zingiberaceae, Myristicaceae and Sapindaceae, and even single genera in one

family, have evolved the same mechanism of a third integument developing over the fertilized ovule? I can find no answer to this. There is no means of evolving an aril *de novo*.

b. The early, intermediate, state with a slight beginning of an aril and an undetached seed could have no survival value. Thus an undetached seed in the rain-forest is almost certain to germinate *in situ* and then to dry up and die before the fruit has fallen off the tree (Fig. 9). And yet there are probably more instances of rudimentary, useless, arils than of fully developed arils.

c. Why should *Sloanea* be the only arillate capsule of the Tiliaceae–Elaeocarpaceae, and yet agree in this fruit so closely with the allied Bombacaceae and Sterculiaceae?

d. Why should the Myristicaceae, with very reduced and simplified flowers, have evolved this massive fruit as a universal peculiarity of their peculiarly isolated family? Their big arillate seed is, indeed, a handicap in that it is the chief factor preventing their migration from the tropics, because this seed has no power of dormancy.

B. On the other hand, *if the large arillate fruit is a relic*, then one can easily understand:

a. That most flowering plants have passed on to other kinds of fruits with smaller seeds and better, or more xerophytic, dispersal mechanism in drupes, nuts, achenes, winged seeds, and so on: particularly must this have been necessary for herbaceous plants which cannot possibly reproduce by large arillate fruits. The rarity, then, of the arillate fruit follows from its primitive nature as the unspecialized means of reproduction of tropical rain-forest trees.

b. That there are many useless vestigial arils as relics.

c. That *Sloanea* is a connecting-link between Elaeocarpaceae, Bombacaceae, and Sterculiaceae in its arillate capsule.

d. That only those flowering plants could spread out of the tropical rain-forest which had evolved fruits and seeds better adapted to drought and cold than the large arillate fruits with their exposed, fleshy seeds. Thus the Myristicaceae appear as the one family of tropical trees which have been unable to spread into the monsoon and temperate regions, because they have been unable to evolve a new kind of fruit.

(Compare *Dysoxylon* and *Melia*, Bombacaceae and Malvaceae, Elaeocarpaceae and Tiliaceae, Swartzioideae and Papilionaceae, Dilleniaceae and Ranunculaceae, *Bocconia* and *Papaver*, Scitamineae and Liliaceae, &c.)

C. *Conclusion.* The red, fleshy, and often spiny follicle or capsule, with large black seeds covered by a red or yellow aril and hanging from the edges of the fruit-valves, is the primitive fruit of modern flowering plants.

In many families it is easy to see how this fruit has changed into the dry follicle or capsule with small, often winged, easily detached, exarillate seeds, or into the berry, drupe, and nut because there are many intermediates in existence. As an instance I will outline the state in the Leguminosae.

LEGUMINOSAE

Arillate genera. There are in the following four sub-families eighteen genera with the aril more or less covering the seed:

Mimosoideae: 2 genera out of *c.* 50 (*Acacia*, *Pithecellobium*).

Caesalpinioideae: 14 genera out of 126 (or 70 species out of 2,300).

Swartzioideae: 1 genus out of 9.

Papilionaceae: 1 monotypic genus out of *c.* 500 genera and 10,000 species.

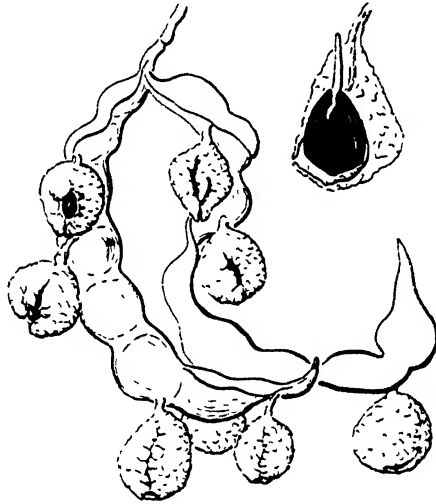


FIG. 9. A dehiscent legume of *Pithecellobium dulce*, showing the hanging black seeds covered with the rose-red aril ($\times \frac{1}{2}$); a seed germinating in the aril, undetached from the pod (the aril in section) ($\times 1$).

This is obviously a relic distribution, and nearly every arillate genus shows in different species all stages in reduction or loss of the aril. If the exarillate state were primitive, one would expect to find the inverse proportion, but what is abundant now cannot possibly be primitive (cf. Cycadaceae compared with Abietaceae, Dilleniaceae with Ranunculaceae; or *Amphioxus*, *Peripatus*, and *Monotremata*; or the elephant, tapir, and anthropoid apes, as relics proved by fossil record).

Pithecellobium. *P. dulce* has the black seeds entirely covered by the red aril, and the wall of the pod is pink and somewhat fleshy. Seeds, uneaten from the pod, often germinate *in situ* to wither up through lack of water. *P. ellipticum* has large black seeds which hang from the red pods on long funicles, but they have no aril at all: the seed-coat is thinly pulpy (sarcotesta) and is eaten by birds. In *P. clypeatum* there is no aril or pulpy seed-coat, and the pod opens only in the places where the seeds are, not in the intervals between the seeds, yet it still has the red colour and vivid red inner face (as in *Xylopia*, *Sloanea*, *Sterculia*, &c.). Other species have indehiscent pods. The genus clearly shows a transition from the rare arillate pod to the common

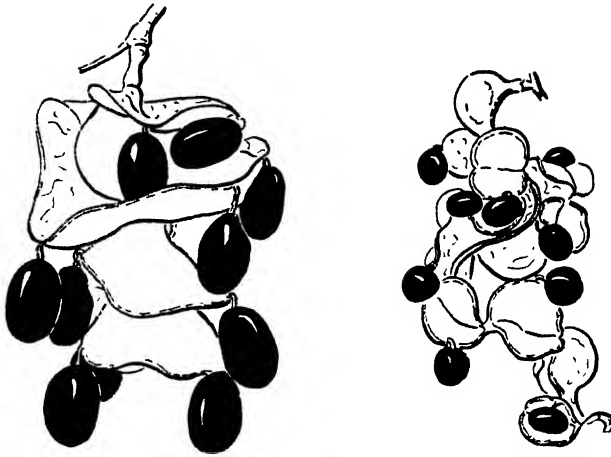


FIG. 10. Dehiscent red legumes of *Pithecellobium ellipticum* (left) and *P. clypeatum* (right), showing the black seeds hanging on persistent funicles (red in *P. ellipticum*) but without arils; the pods of *P. clypeatum* opening only in the parts containing the seeds ($\times \frac{1}{2}$).

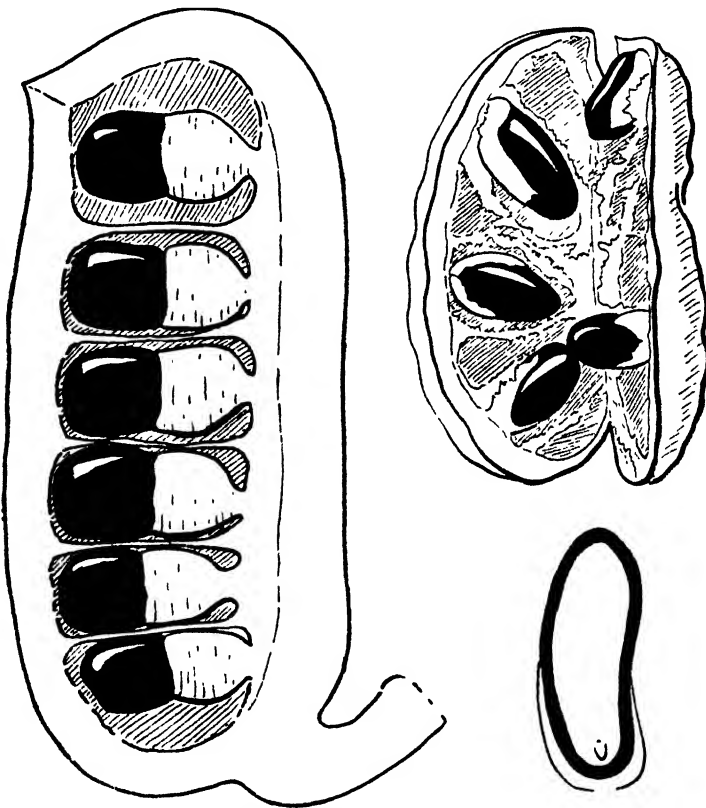


FIG. 11. A mature legume of *Pahudia cochinchinensis* (left, Caesalp.) with one valve removed, showing the red arillode and black seed ($\times \frac{1}{2}$) (from Pierre, Fl. For. Coch. Ch., t. 386); a dehiscent legume of *P. javanica* (right, $\times \frac{1}{2}$), and a seed with red aril in section ($\times 1$) (from Prain, Ann. Roy. Bot. Gdn. Calc., ix, 1901, t. 44).

Mimosa-condition with small dry seeds or the indehiscent state which cannot in any way be considered primitive and upgrade.

Caesalpinioideae. Well-developed arils occur in *Copaifera* and *Pahudia*, but commonly there is no aril and, instead, the funicle becomes fleshy as an arillode, e.g. *Intsia* (allied with *Pahudia*) and *Sindora*. *Tamarindus*, *Hymenaea*, and *Detarium* have woody, indehiscent pods but have distinct arillodes as well developed as in the dehiscent pods of *Intsia* and *Sindora*: but, in so far as

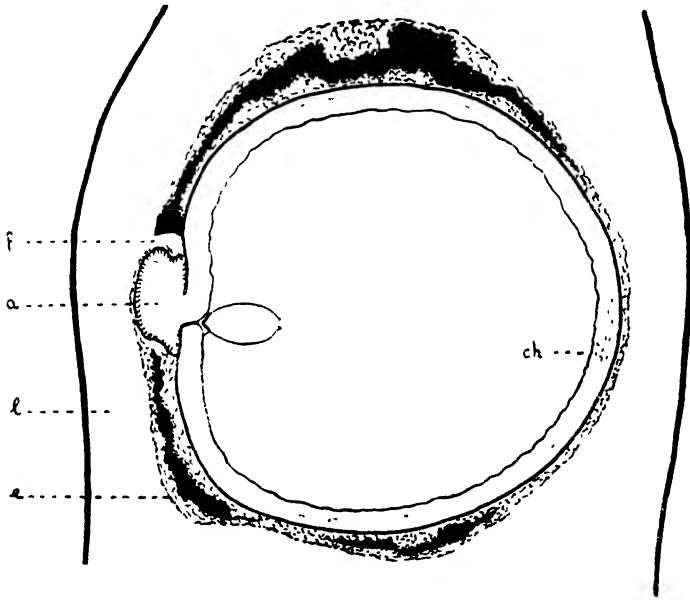


FIG. 12. A mature seed in longitudinal section in the legume of *Intsia bijuga* (Caesalp.), one cotyledon filling the space of the seed ($\times 2$). *a*, arillode; *ch*, chalaza; *e*, the mealy endocarp, partly stuck on to the seed and partly disrupted; *f*, funicle; *l*, the wall of the legume.

these arillodes are never exposed to view, they are functionless as such and cannot be upgrade. Indeed, in *Detarium*, the pod has become a drupe with a stone so hard that it must be cut with an axe, and in a nook on the inside of this stone the arillode is hidden: both pods and arils are clearly down-grade.

Arillaria robusta. This rare monotypic genus of lower Burma and Siam is the only member of the Papilionaceae to have a fleshy pod and a black seed entirely covered by a red pulpy aril. In all other respects the genus resembles the pantropical *Ormosia* with *c.* 50 species. *Arillaria* is, for me, not an oddity but a priceless relic, such as *Amphioxus* or *Ginkgo*, which proves what has been the ancestral fruit of the Papilionaceae. Indeed, *Arillaria* in this respect recalls those other three relic monotypic genera of the Caesalpinioideae, namely, *Tamarindus* (India), *Amherstia* (Burma), and *Lysidice* (South China, Indo-China), which show what an enormous variety of magnificent flowering trees must have become extinct in the evolution of the Caesalpinioideae.

Papilionaceae. Nevertheless, if they lack big red arils, still many *Papilionaceae* have small horny greenish, yellowish, or white arils surrounding the hilum as a rim, e.g. *Mucuna*, *Tephrosia*, *Cytisus*, or *Lathyrus*. Indeed, I have found this rim-aril present, if microscopic, in every *Papilionaceous* seed that

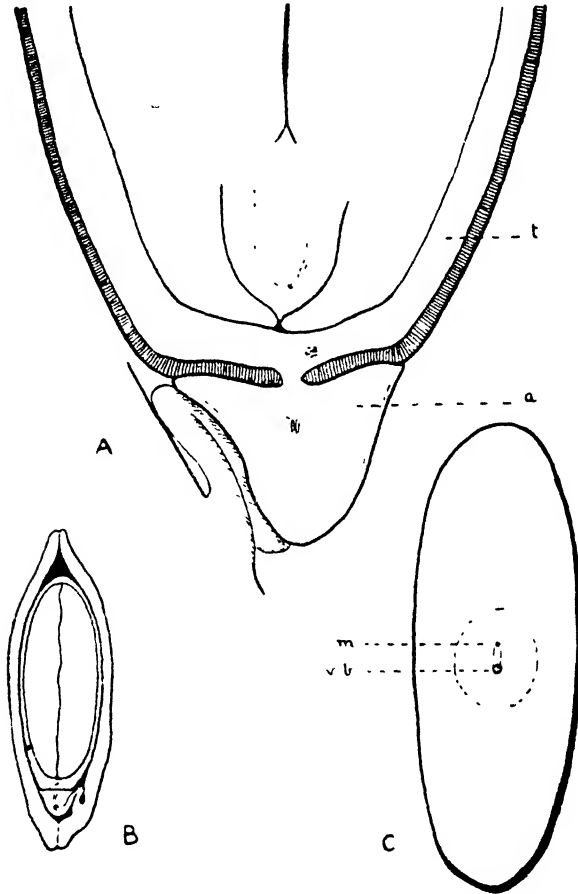


FIG. 13. Details of the seed of *Intsia bijuga* (Caesalp.). A, the base of the seed in trans-median longitudinal section, showing the long recurved funicle, the pink, hard, corticated arillode, and the testa with its palisade ($\times 6$); B, a ripe pod in transverse section, showing the seed *in situ* ($\times 1$); C, a mature, detached seed in hilar view, showing the faint arillode-scar ($\times 2$). a, arillode; m, micropyle; t, testa; v.b., vascular bundle.

I have examined (with the exception of *Inocarpus*), and I conclude that every *Papilionaceous* hilum has, or had, a *rim-aril*, at least, if not a fully developed aril. In other words, the dry rattling pod is the modern shadow (very efficient, no doubt) of the fleshy arillate pod. The arillate seed has no survival-value in the modern *Papilionaceae*.

Adenathera, *Ormosia*, *Erythrina*, *Abrus*. These four genera, the first being Mimosoid, the others *Papilionaceous*, have hard red seeds which hang on the dry opened pods with persistent funicles, but no aril. Why? As with the

durian, so with these beautiful objects, there is an evolutionary history to account for their oddity. What is it?

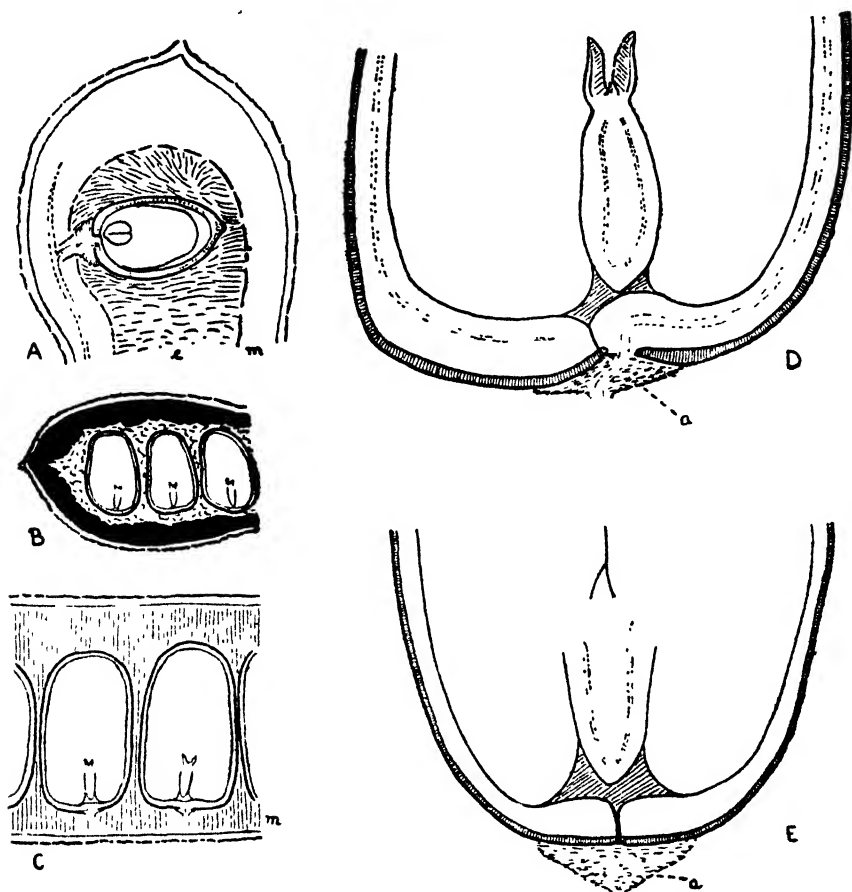


FIG. 14. Details of the seed and indehiscent legume of *Tamarindus indicus* (Caesalp.) showing the functionless white powdery aril at the base of the massive seed. A, the apex of a young legume with immature seed (the endosperm encroaching upon the nucellus), but with the aril developed, the cavity of the legume filled with the cottony-pulpy hairs of the endocarp e ($\times 2$); B, the apex of a ripe legume, showing the shell-like exocarp and the pulpy mesocarp contracted into a sticky brown mass on the seeds, leaving a gap (black) between them and the epicarp ($\times 1$); C, part of full-grown, but unripened, legume (in l.s.) showing the pulpy mesocarp m ($\times 1\frac{1}{2}$); D, E, the bases of full-grown seeds in median and transmedian longitudinal section, and the thick testa with external palisade ($\times 6$). a , aril; e , endocarp; m , mesocarp.

In *Adenanthera bicolor* and some species of *Ormosia* and *Erythrina* the seeds are partly black and partly red. The red part is that near the hilum and micropyle, the black being at the chalazal end. It seems that there has been a *transference of function* (see Corner, 1949): the aril has been lost (*Adenanthera*) or reduced to a rim-aril (Papilionaceae), but its redness has been transferred to the seed by invasion from the funicular end where the aril normally develops. Thus, these half-and-half seeds are a step between the black seed

with red aril and the red seed without aril. Black-and-red seeds, few and comparatively rare, are relics. But, in *Abrus*, an inversion seems to have occurred, perhaps as a mutation on a normal sequence, for the red part of the seed is chalazal and the black part is round the hilum. In *Erythrina*, too, there

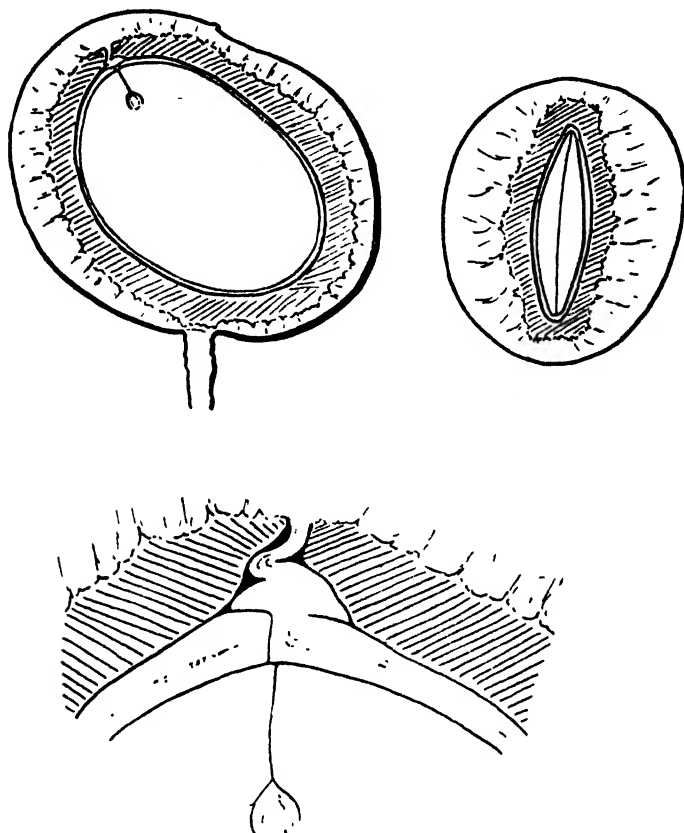


FIG. 15. The ripe drupe-like follicle of *Detarium senegalense* (Caesalp.) in median and trans-median longitudinal section, showing the hard stone (endocarp) with fibres entering the pulpy mesocarp, and the minute, pale-pink functionless aril inside the indehiscent endocarp (upper figures $\times 1$; lower figure $\times 5$).

are some anomalies. However, there is clearly something to be learnt even from bi-coloured seeds.

The red seeds, hard though they are, are eaten plentifully by strong-beaked birds—they catch the parrot's eye and, unless he cracks them, their seed-coat is so hard they will not germinate.

A parallel example is *Guarea* (Meliaceae) with red seeds but no aril, superficially exactly like the seeds of *Dysoxylon* (Meliaceae) which are wholly covered by a red aril. In each case the red colour is in the cuticle.

Arillodes. The fleshy red, pink, yellow, or white funicle is called an *arillode* (see Pfeiffer, 1891). It represents clearly the long Mimosoid and Caesalpinoid

funicle which has had transferred to it the function of the aril, while the aril has disappeared. Fig. 20 shows how easily the characters of the aril may be displaced and transferred. Thus, the aril develops in the Leguminous seeds

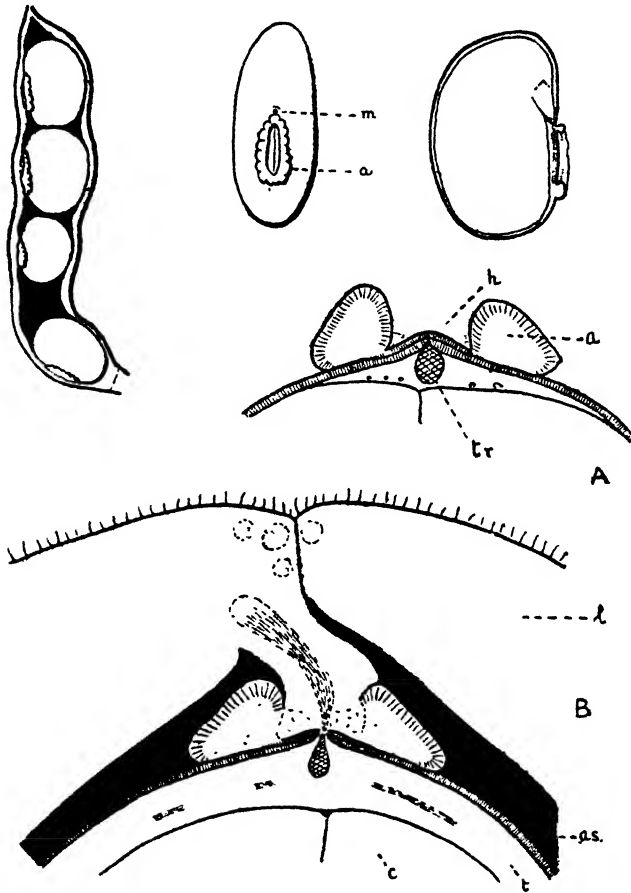


FIG. 16. A mature legume (one valve removed, $\times \frac{1}{2}$) and seeds (one in section, $\times 1$) of *Mucuna utilis* (Papilion.) showing the rim-aril surrounding the hilum (an oblong socket). A, a transverse section of the hilum of a ripe dried seed showing the rim-aril attached to the palisade of the testa by the counter-palisade of the hilum ($\times 7$); B, a transverse section of the hilum and funicle of a full-grown, but unripened, seed, showing the aril as the dilated head of the funicle, the vascular bundle of the funicle contacting the tracheide rod of the hilum, and the funicle breaking off in the aerenchymatous tissue in its head ($\times 7$). a, aril; a.s., air-space; c, cotyledon; h, hilum; l, the legume-wall; m, micropyle; t, testa; tr., tracheide rod of the subhilum.

from the region marked A. A slip in time or place of differentiation of the aril-characters may transfer them to c, which is the funicle, and then the arillode is formed. Thus, in *Acacia*, there are numerous transitions from arillate seeds with long funicles to seeds with arillodes (Fig. 21). Further displacement to the placenta (D) will produce the red pseudo-arillate placental sacs which

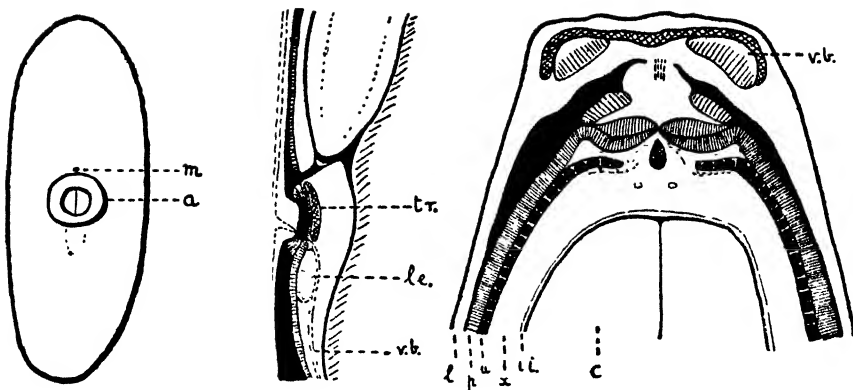


FIG. 17. Seed and hilar structure of *Desmodium triflorum* (Papilion.), showing a seed in hiliar view ($\times 25$), and a longitudinal ($\times 25$) and a transverse section ($\times 50$) of the hilum of the seed still attached to the fruit-wall, the air-spaces being shown in black. The seed breaks from the funicle by rupture of the aerenchymatous tissue in the head of the funicle, the counter palisade of which remains fixed to the palisade of the testa in the hilum (see Fig. 18). *a*, rim-aril; *c*, cotyledon; *i.i.*, the inner integument; *l*, the wall of the legume; *le.*, the lens of thick-walled cells in the testa; *m*, micropyle; *p*, the external palisade of the testa; *tr.*, the tracheide rod of the subhilar tissue; *u*, the hour-glass cells of the testa; *v.b.*, vascular bundles; *x*, the cortex, or body, of the testa.

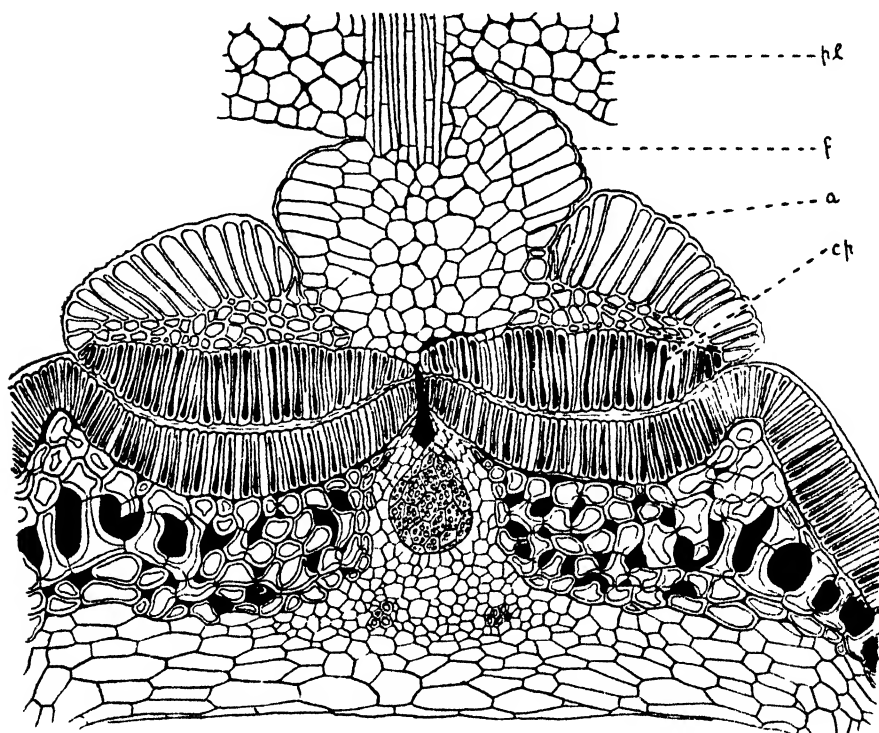


FIG. 18 A transverse section of the hilum of the seed of *Desmodium triflorum*, still attached to the funicle, as typical (in miniature) of the Papilionaceous seed with rim-aril ($\times 225$). *a*, aril (a single layer of elongate epidermal cells); *c.p.*, the counter palisade of the head of the funicle, stuck to the palisade of the testa; *f*, the funicle; *pl.*, the placenta; the subhilar tissue with tracheide bar and two recurrent vascular bundles: the air-spaces of the aerenchyma in black.

envelop the seeds of *Momordica* (Cucurbitaceae) or the red placental mush which covers the seeds of *Randia* spp. (Rubiaceae) and, possibly, *Pittosporum*: and finally, on displacement to the endocarp (E) the red pulp of berries or, changing red for yellow, the pulpy papaya which often has abnormal arillode-funicles, and, eventually, the tomato and the orange.

Red fleshy seeds. If the time or place of appearance of the characters of the aril is delayed, on the other hand, and deferred to region B (Fig. 20), they will become the functions of the seed-coat. Hence may well be explained the hard red and the pulpy red seed-coats in genera and families with arillate allies.

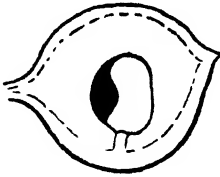


FIG. 19.

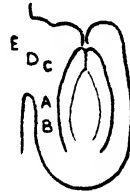


FIG. 20.

FIG. 19. A black and red seed of *Ormosia* sp. (Papilion., collection Black 48-2668, Inst. Agronomico do Norte, Brazil), with black chalazal part: the legume 1-seeded. ($\times 1$.)

FIG. 20. A diagram of an anatropous ovule. A, the region of the aril, B of the testa, C of the arillode (funicle), D of the placenta, and E of the endocarp.

As the red seeds of *Adenanthera* follow, as it were, from the black seeds with red arils of *Acacia* and *Pithecellobium*, so the red seeds of *Iris foetidissima* and *Gloriosa superba*, on persistent funicles in dry loculicidal capsules, indicate that the Liliaceae had an arillate ancestor, as do also the red berries of *Draecena*; and the proof is given by the relic-arils in *Colchicum* and *Asphodelus*. Similarly the red pulpy seeds of Magnoliaceae relate to the arillate seeds of Annonaceae, Dilleniaceae, and Myristicaceae, and the red pulpy seeds of many genera of Euphorbiaceae (*Sapium*, *Glochidion*, *Aporosa*, *Cheilosa*, *Baccaurea*) relate to the rare arillate seeds of the family, exactly as *Garcinia* (pulpy seed-coat) does to *Clusia* (arillate) in the Guttiferae. *Bixa* has both a red pulpy seed-coat and a rudimentary aril.

Three more relics. The genus *Delonix* (Caesalpinioideae) consists of two species of east Africa and peninsular India. This is a well-known relic, or Lemurian, distribution. *D. regia*, the Flame of the Forest, is limited to Madagascar and was nearly extinct when it was discovered in 1830. Now it survives widely, as an ornamental, because its brilliant red flowers present in their symmetry a primitive grandeur. The fruit has not yet been adequately described. It is a massive, dirty-brownish, sabre-like, dry pod, 40-60 cm. long, gaping slightly but enough to allow some 60 dull grey seeds, *c.* 2 cm. long, to hang out on persistent funicles for weeks until these have decayed. This sordid object, revived durianologically, becomes a scarlet sabre, 2 feet long, of black seeds with red arils, and shows what has disappeared from the face of the earth. If not, why does *Delonix* have this fruit?

The genus *Archidendron* consists of some twenty species in Austro-Malaysia. It is a Mimosoid genus characterized by having 5–15 carpels in the flower. It appears, therefore, to have the most primitive gynoeceum of the

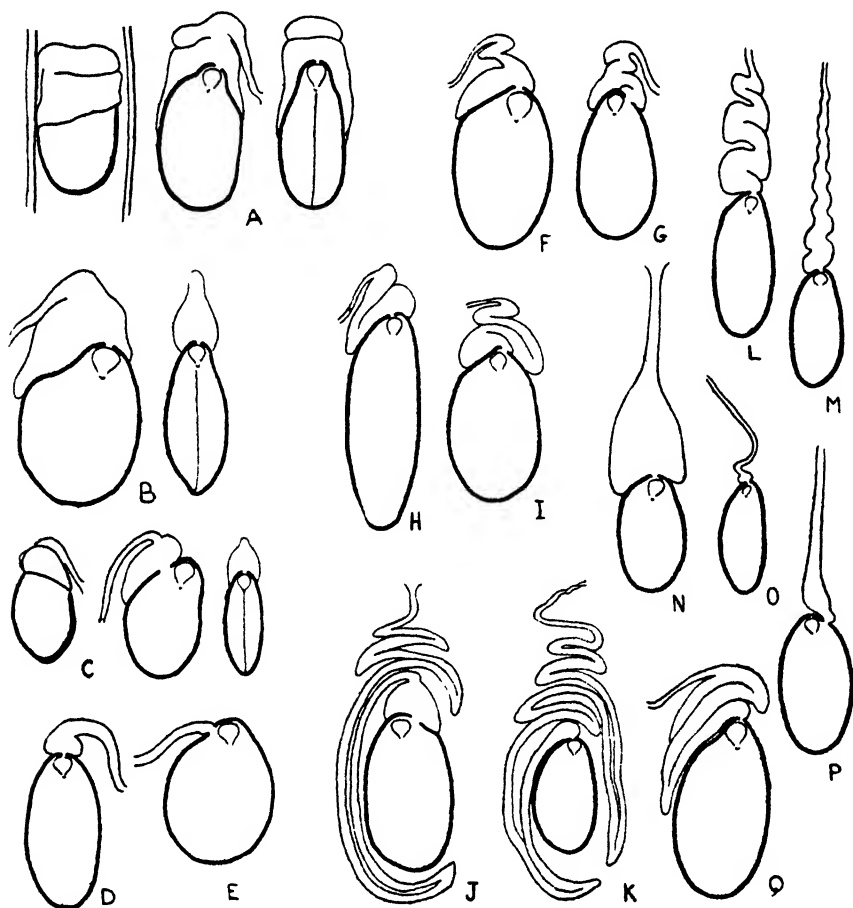


FIG. 21. Seeds of *Acacia* (copied from Mueller, Ic. Austral. Sp. Acac., 1888), mostly in median longitudinal section, to show the reduction of the aril or its modification into the arillode ($\times 1$). A, *A. colletioides*, 3 seeds (one in the pod), showing the well-developed aril, disappearing in B–E; B, *A. sessiliceps*; C, *A. coriacea*; D, *A. latifolia*; E, *A. praelongata* (with simple funicle); F–K, showing the elongation of the funicle and loss of aril; F, *A. phlebocarpa*; G, *A. Wallachiana*; H, *A. Luehmannii*; I, *A. lysiphloia*; J, *A. anceps*; K, *A. cincinnata*; L–Q, showing the development of the arillode, or fleshy funicle, with loss of aril; L, *A. stipuligera*; M, *A. aulacocarpa*; N, *A. retivena*; O, *A. gonoclada*; P, *A. delibrata*; Q, *A. montana*.

Leguminosae. What kind of fruit does it have? From each flower, at least in the Australian species, develops a bunch of large, red, fleshy pods with yellow interiors and many large, black, shiny seeds hanging on long funicles (see the illustration, Bailey, 1916). There seems to be no aril, but, from the examples of *Sloanea*, *Durio*, *Sterculia*, *Acacia*, &c., I have no doubt that at least one species will be found with an aril. The fruit of *Archidendron*, as well

as the gynoecium, is thus extremely primitive and is the living proof of the pathetic decadence in the beautiful *Delonix*.

The long funicle (1-6 cm.) for the dangling seed is characteristic of the Mimosoideae, especially, the Caesalpinioideae and the Swartzioideae, in contrast with the Papilionaceae. The Asiatic species of *Parkia* (Mimosoideae)

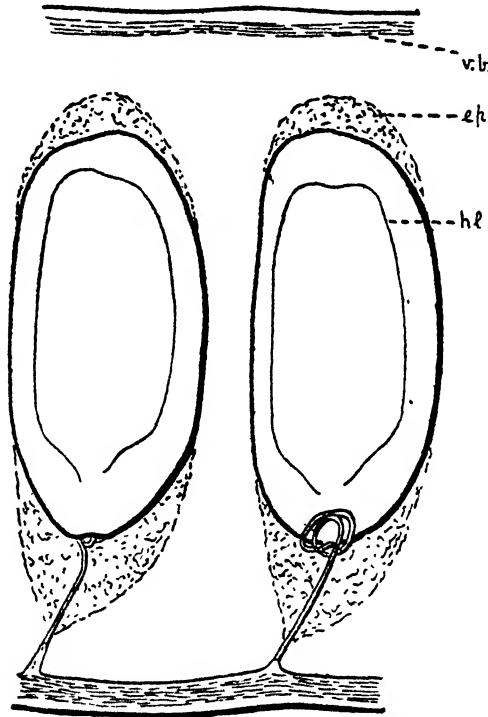


FIG. 22. Two ripe seeds of *Parkia javanica* (Mimos.) in the indehiscent legume, showing the long but functionless funicles ($\times 2$). *ep.*, the endocarp-pith; *hl.*, the heart-line of the testa (characteristic of many Mimosaceous seeds); *v.b.*, the vascular bundle on the dorsal side of the legume.

have indehiscent pods containing rows of large seeds with long, slender, coiled funicles. Why? The length of the fine funicle is useless and clearly a cause of the long delay in ripening of the pods by reducing the food-channel to the seeds. But, as a characteristic of the dangling arillate seed of *Acacia*, *Pithecellobium*, and *Swartzia*, it is readily understandable as a relic. The pods are dehiscent in some of the tropical American species of *Parkia* and the hanging seeds are eaten by parrots. Indeed, the red macaw appears to feed largely on a red-flowered *Parkia* of the Amazon valley.

The bunch of *Parkia*-pods at the end of the swollen clavate head of a long stalk resembles an elongate head of *Archidendron*: but, instead of being the production of a single flower, it is the multiple product of the unicarpellary flowers in the centre of the *Parkia*-capitulum. Thus *Parkia* represents the

state of *Archidendron* in the second degree, as a Composite-capitulum is a second-degree flower. Both are caused by intricate transference of function in embryonic structures. But it is interesting to see how this isolated genus of tropical trees with capitulate inflorescences of highly reduced flowers (the lower, even, sterile and 'attractive' as in the Compositae) retains the essential characters of the cluster of arillate pods, as a primitive determination.

Conclusion. The primitive Leguminous fruit was a cluster of large, many-seeded, red fleshy pods with black seeds, each covered by a red aril and hanging out on a long funicle. Perhaps the pods were spiny, a half to one metre long, with some 50 seeds, and may even have been held erect (cf. *Pentaclethra*).

Corroboration. The only family which is at all nearly related with the Leguminosae is the Connaraceae. Many Connaraceous genera have red pods and black seeds with red or yellow arils (mostly covering the lower part of the seed). Some genera, as *Cnestis*, have 5 carpels thus developed from the flower, and their fruits resemble those of *Sloanea* and *Sterculia*. Perhaps the Annonaceae are related: at least, in *Xylopia* one finds the same apocarpous polycarpellary ovary developing a cluster of arillate follicles. But the evidence goes to show that the Leguminosae are one of the most isolated series of flowering plants and should constitute by themselves one of the main subdivisions of the Dicotyledons.

OTHER EXAMPLES

Bocconia. I saw near Bogotá, in 1947, across a valley some pinnate leaved treelets resembling palms. Dr. Enrique Perez-Arbelaez, the Colombian botanist, told me that they were saplings of the Papaveraceous genus *Bocconia*, the two species of which develop into trees up to 10 or 15 m. high. I said on reflection, at first hearing of a tree-poppy, that it must have rather large black seeds with red arils, though I was acquainted only with the minute seeds of *Papaver* and its allies. Dr. Perez-Arbelaez remembered that it had; and, shortly, we found the fruits. They are rather small, yellowish, fleshy, loculicidal capsules, c. 12×7 mm., containing 1 (rarely 2) black seeds, 7×3 mm., with a red aril round the base: the seed hangs from the persistent hoop-like replum (Fig 23). The deduction was proved, and affords one of the most striking evidences of the Durian theory that I have met. One could have argued back from the minute arils, or strophioles, in the seeds of *Chelidonium* and *Fumariaceae*, but *Bocconia* is the living relic.

Aesculus. The spiny, loculicidal, fruits of the Horse-chestnut (*Aesculus*), with large brown seeds and thick white endocarp, resemble in so many ways a small durian that, in 1946, I carefully studied developing fruits to see if there was a trace of an aril. I found none, but Dr. Dugan, Professor of Botany in the University of Bogotá, informed me that the Colombian and Central American genus *Billia* had arillate seeds, though its fruits were spineless.

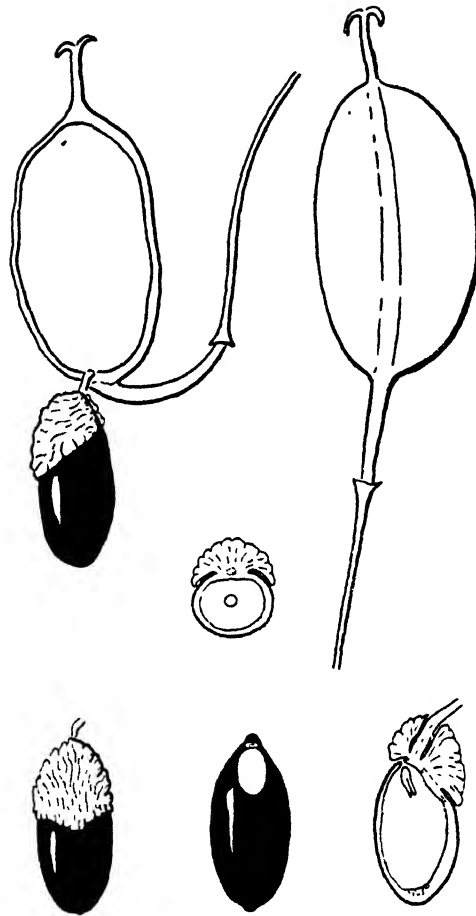


FIG. 23. Fruits and seeds of *Bocconia frutescens* (Papaveraceae) ($\times 3$). an indehiscent and a dehiscent fruit with the valves fallen from the replum and the single seed dropped out on the funicle. the black seeds with red aril, the central lower figure showing the white patch where the aril is attached.

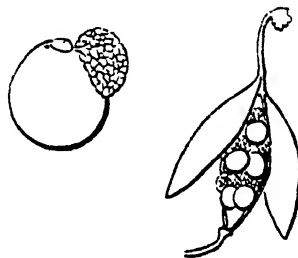


FIG. 24. Fruit and seed of *Corydalis lutea* (Fumariaceae), showing the rudimentary aril, mag. (from Payer, *Traité d'Organogénie*, 1857, t. 50, f. 14 and 15).

PRIMITIVE ANGIOSPERMS

For the immediate ancestors of modern flowering plants, I postulate, therefore, massive red follicles or capsules with many large black seeds with red arils. It is possible from this startling premiss to make the following deductions:

1. *Trees*. Such fruits must have been borne on massive twigs. No herb could produce a durian, a nutmeg, or an Annonaceous fruit, far less the Leguminous cluster of arillate pods. Therefore it must be inferred that these ancestral flowering plants were *trees*.

2. *Tropical*. Large arillate seeds have no power of dormancy or of withstanding desiccation and cannot survive outside tropical and sub-tropical rain-forests. Therefore the ancestors must have been *tropical trees*.

3. *Compound leaves*. Tropical trees with massive twigs are typically those with massive, compound spirally arranged leaves, whether pinnate or palmate, and in such trees there are always transitions to simple leaves. Therefore these ancestral tropical trees must have had *compound leaves* (pinnate in the first place, for the palmate is the pinnate with reduced axis).

Such is the present-day condition of many trees with arillate fruits borne laterally on the current shoots, that is, in the leaf-axils, e.g. Meliaceae, Sapindaceae, Leguminosae, Connaraceae, *Sterculia*, *Bocconia* (*Aesculus*).

4. *Monocauly*. The following two complementary principles occur with such regularity in the construction of flowering plants as to appear susceptible of mathematical treatment:

a. *Axial conformity*. The stouter, or more massive, the axis in a given species, the larger and more complicated are its appendages. Thus the stouter the main stem, the bigger the leaves and the more complicated their form, e.g. saplings of trees (some with compound leaves while the branches have simple leaves, as in *Artocarpus*, *Scaphium*, and some Proteaceae), or the stems of herbaceous plants as *Nicotiana* and *Helianthus*, or rosette Umbelliferae and Compositae with the large basal leaves diminishing in size and form to bracts.

b. *Diminution on ramification*. The greater the ramification, the smaller become the branches and their appendages, e.g. in *Solanum*, the leaves, inflorescences, flowers, fruits, and twigs become smaller as the ramification increases; and, in *Carica papaya*, the scarcely branched female inflorescences have a few large flowers whereas the highly branched male inflorescences have many small flowers.

These principles are needed to understand what form a primitive angiosperm must have taken in order to produce the enormous fruiting mass which must be postulated, and to understand how the modern angiosperms, highly ramified with slender appendages, have thence been evolved. The two principles, it may be noted, do not apply to algae, having more or less wholly assimilatory surfaces, but are new principles of Xerophyton giving to land-plants their regular diminishing, not expanding and thallus-like, appearance.

Now, reduce a pinnate-leaved flowering tree to an undivided stem, that is, its sapling stage, and turn the apical bud into a single terminal flower. The result will be clearly a Cycad-like tree with a rosette of sterile and fertile floral leaves, and such must have been the appearance of the proto-Leguminosae with the terminal rosette of gigantic arillate follicles. Indeed, this proto-Leguminous follicle so closely resembles the Cycad megasporophyll that it is necessary merely to regard the red pulpy seed of *Cycas* as a transformed arillate seed (as of *Taxus*) and to incurve the sporophylls to form a follicle (as by the common process of precocious maturity) in order to arrive at once at the proto-Leguminous follicle.

Now, reverse this procedure. Make the flower axillary and it will become smaller, though still massive (e.g. the flowers of *Magnolia*, or the female flowers of *Carica*). Then ramify the axillary buds to produce highly branched inflorescences, and the flowers will be yet smaller (as in the cymes of *Annonaceae*, or the male inflorescences of *Carica*, some of which still retain a single massive terminal female flower). Finally, ramify the stem and produce ultimately the very small flowers of the panicles of *Sapindaceae*, *Meliaceae*, *Mimosoideae*, and other modern trees with twigs which are slender compared with their sapling stems. The modern solitary Leguminous carpel is, thus, the still more precociously matured proto-Leguminous carpel fitting the bud-character of the relatively small modern Leguminous flower borne on an ultimate ramification of high degree: and the modern solitary Leguminous follicle represents the Cycad megasporophyll developed *post*, instead of *pre*-, fertilization (see Corner, 1949), as all that the relatively slender axis can bear in the way of fruit. As the Cycad megasporophyll unfolds, the Leguminous follicle ultimately dehisces, and, as the Cycad megasporophyll must be borne on a massive stem, so too the peduncle of the Leguminous flower thickens and matures *post*-fertilization. Thus, it can be understood that, to fulfil their hereditary requirements, fertilized ovules had to enlarge into seeds and fruits had to enlarge and dehisce: and so, too, at the other extreme, it can be understood how the modern Compositae, for example, have escaped this hereditary yoke, the achene showing practically none of the features of the follicle and capsule. Seed-structure and seed-demands will be found more and more important in the study of flowering plants.

By arguing back to the single stem, or monocaulous state, with huge open terminal flower, as the necessary progenitor of the huge fructification demanded by the Durian theory, one arrives not at a figment but at a reality, the well-known *Cycas*, which has so often posed as the angiospermous prototype. Now, it can be seen how, without entering into particular detail, the modern Dicotyledonous tree may well have been evolved from this prototype by ramification in all parts, leading to the diminution in the size and the complexity of structure of branches, leaves, flowers, fruits, and seeds, and by relegation of flowers to axillary buds: but the fruit tends to revert to the ancestral form of the Cycad megasporophyll because the seed, as the dispersal organ, is the conservative and overruling factor in reproduction.

Typically the Cycad is devoid of internodes. The large leaves dominate the stem-apex and the stem itself, as in the tree-ferns. It would seem that the tendency to produce internodes is a new process of juvenescence running through the evolution of angiosperms and ending in the appearance of the herb, that is, the tendency to prolong the seedling phase and so to produce elongated stems at the expense of the development of the leaf-base, rather than the leaf-rachis. The Cycad-phase of angiosperm-evolution, however, is clearly shown with the addition of incipient internodes in the growth-form of *Carica papaya*, palms, pandans, and the saplings of trees with compound leaves in general (Araliaceae, Caesalpinioidae, Bignoniaceae, and even by such Euphorbiaceae and Annonaceae as *Phyllanthus* and *Drepananthus* the phyllomorphic ramuli of which retain this ancestral trait to the second degree of branch-systems with simple leaves, the whole resembling compound leaves). The terminal inflorescence of pandans and of Bignoniaceous trees, enforcing sympodial growth, appears as a primitive character, in that the axillary inflorescence has not been evolved. Similarly, the massive rosette of *Agave* with its terminal inflorescence and monocarpic habit appears as an immediate derivative of the Cycad form and more primitive than the ramified trees of *Dracaena*. Among palms, the sequence can be read from monocarpic palms, with huge terminal inflorescences (*Corypha*, *Metroxylon*) and immense leaves, to highly branched palmlets (*Bactris*, *Geonoma*, *Pinanga*) with slender stems, small leaves, and lateral inflorescences. It can be seen, therefore, that neither has the ramification of the stem proceeded *pari passu* with that of the inflorescence, nor have these factors been linked necessarily with the transference of the terminal inflorescence or flower to the lateral position: thus, no Monocotyledon really corresponds with *Magnolia* or *Nymphaea* in having massive, solitary, axillary flowers, although there are many higher analogues such as sympodial trees or unbranched rosettes with terminal inflorescences (*Agave*, *Lobelia*).

Monocauly, therefore, and monocarpy appear not as newly acquired peculiarities of modern plants but as relics of the normal features of the early angiosperms. Conversely, the highly branched tree with slender twigs, simple leaves, and highly branched inflorescences of very small flowers, typical of the Amentiferæ, appears in its true light as a modern derivative. Furthermore, the apparent oddities of flower-size, inflorescence, and sex in *Carica* are seen to represent a phase in the evolution of the axillary inflorescence which most other Angiosperms have undergone.

Note. Can *Carica papaya* be induced to form a terminal flower? Its naked bud is open for experiment, and its massive stem offers the possibility of producing a proto-angiospermous flower, by reversion, at its apex. It is a very generalized plant of primitive habit, surviving, no doubt, by its chemical virtues rather than its structural.

5. *Leptocauly*. I use this name to indicate the modern tree with relative slender primary axis and branches in contrast with the *pachycaulous* Cycad. Increasing ramification, the evolution of the simple leaf, and the development

of internodes are the basic features of the modern tree. The slender twig, with long internodes, provides length, or height, with less weight, and leads not only to the rapid overtopping of the old, clumsy pachycaul with massive and slow-growing branches, but outstrips it also geographically by providing the leptocaul with better powers of resisting drought or cold, if only because the small buds are more numerous and more easily made and the damaged twigs more easily substituted. The leptocaul, or modern tree, thus comes to dominate in height and spread and distribution the ancient pachycaul, forming the modern forests, while the palms, pandans, *Carica*-like trees, and so on, just as the Cycads and tree-ferns, are relegated to subordinate and, as their constitutions still require, mainly tropical stations.

6. *Cauliflory*. If a highly advanced leptocaul retains the old massive flower and fruit, as the Durian theory demands, then its flowering and fruiting must be postponed for dormant buds on matured wood, the slender leafy twig being too precocious. Thus are introduced ramiflory and cauliflory according to the degree of ramification and relative physiological immaturity of the twigs and branches. Most species of *Durio*, *Xylophia*, and *Myristica*, for example, with slender twigs and simple leaves in applanate sprays, but with massive arillate fruits, are ramiflorous and cauliflorous: and, even, pinnate-leaved trees with slender twigs and applanate foliage, as *Swartzia* or *Lansium*, but with massive arillate fruits, become ramiflorous or cauliflorous.

The *tropical* phenomenon of cauliflory receives, therefore, a simple and natural explanation as the instance of trees which have evolved the modern twig but have retained the old habit of the arillate fruit. This massive fruit implies, however, a massive flower or inflorescence or, at least, a physiological massiveness as an advanced state of maturity of the tissues before the reproductive organs can be developed: and, while the arillate fruit may have passed on to the indehiscent drupe, berry, or nut, either the massive flower or inflorescence or the physiological requirements remain to enforce cauliflory, as in *Annona*, *Polyalthia* (Annonaceae), *Averrhoa* (Oxalidaceae), *Diospyros* (Ebenaceae), or *Theobroma*.

An instructive example is afforded by *Artocarpus*. *A. anisophyllus* has the largest, spirally arranged, pinnate leaves and the most massive twigs of the genus, and its large fruits are axillary. *A. incisus* (Breadfruit) has almost as massive, pinnatifid leaves and twigs, but the fruits tend to mature on the bare parts of the twigs from which the leaves have fallen. *A. heterophyllus* (the Jack-fruit), however, has slender twigs with simple leaves tending to the modern horizontal spray and is cauliflorous.

Averrhoa, on the other hand, seems exceptional. *A. bilimbi* has massive twigs and is cauliflorous, while *A. carambola* has slender twigs and more or less axillary fruits. As a rule, however, it is not difficult to decide *a priori* from the twig and the flower and fruit whether a tree is cauliflorous.

7. *Megaspermy*. Even the ramiflorous trees of modern form cannot escape from the rain-forests because of their large, quick-germinating seeds. For the colonization of the drier tropics and the temperate regions one must expect

the evolution of cold- and drought-resisting fruits (as drupes and nuts) or small seeds with hard coats and partly desiccated embryos with great powers of dormancy. Thus, trees may again be classified in the two contrasting ways:

Megaspermous trees

Tropical, with large seeds and fruits, not herbaceous.

a. More or less megaphyllous, with rather massive radially constructed shoots and, typically, compound leaves.

b. Ramiflorous-cauliflorous, more or less microphyllous, with slender applanate shoots and, typically, simple leaves.

Microspermous trees

Tropical or temperate: more or less microphyllous and, typically, leptocaul with, usually, simple leaves: with small fruits and small resistant seeds: often with herbaceous derivatives.

Both kinds occur in the tropical rain-forest, apparently in dynamic equilibrium, but the microspermous trees preponderate the more difficult the climate becomes (e.g. *Betula*, *Salix* near the Arctic circle: Ericaceae and small-seeded Myrtaceae on mountains). The dynamic equilibrium exists in this way. The microspermous tree excels by virtue of its leptocaul characters, while the massive-twigged megaspermous tree has the ascendancy in the seedling stage and in the lower layers of the forest. Big seeds, full of food-reserve, give tall plumules raised well above the humus-debris (even to 1 m. in *Dimorphandra* sp. of Surinam, and 3 m. in *Entada* of Malaya), and massive twigs with spirally arranged leaves can ascend the vertical and oblique shafts of light which break through openings in the canopy (Corner, 1946). It is difficult to see how minute seeds can establish themselves on the floor of the forest, except in such places as steep banks or landslips, where the bare earth has by chance been exposed. For the tropical rain-forest, the ideal seems to be the intermediate tree with relatively slender twigs, large seeds and oblique sprays of subspiral leaves, or with the *Terminalia*-habit (see Corner, 1940), such as occur in most Dipterocarpaceae, Sapotaceae, Guttiferae, Lauraceae, Sterculiaceae, Lecythidaceae, Rutaceae, and Leguminosae. However, the tropical forest contains, apparently, all the possible permutations and combinations of tree-characters which independent rates of evolution of the different characters can have brought about, and it will be possible to subdivide the megaspermous trees into many categories as soon as botanists have begun to study them in the forest. Modern tropical rain-forest trees show the adaptive radiation in the building of tree-forms in many families from *Cycas* and *Agathis*, or *Carica* and *Corypha*, to Cupuliferae, Compositae, and *Dracaena*, and such little-known families of trees as Annonaceae, Olacaceae, Rubiaceae, and Euphorbiaceae will repay detailed studies.

Note. The following figures, taken from a large number of examples that I have studied, will give an idea of the range in size of seeds. The weights are averages calculated from samples of 10, 20, 30, or 50 average-sized seeds.

	Average weight of fresh seed (grammes).
<i>Millettia atropurpurea</i> (Papilion.) . . .	60
<i>Carapa guyanensis</i> (Meliaceae) . . .	15.67
<i>Hymenaea courbaril</i> (Caesalpin.) . . .	5.575
<i>Mucuna utilis</i> (Papilion.) . . .	0.891
<i>Parkia javanica</i> (Mimos.) . . .	0.773
<i>Delonix regia</i> (Caesalpin.) . . .	0.407
<i>Cassia fistula</i> (Caesalpin.) . . .	0.151
<i>Phaseolus radiatus</i> (Papilion.) . . .	0.090
<i>Hibiscus esculentus</i> (Malvaceae) . . .	0.065
<i>Cassia siamea</i> (Caesalpin.) . . .	0.0329
„ <i>hirsuta</i> „ . . .	0.0076

Note. *Quercus*, *Fagus*, *Corylus*, *Juglans*, *Aesculus*, *Euonymus*, and *Taxus*, for example, appear to be exceptions, as temperate megaspermous trees. The first four, however, have resistant nuts or stones, thus overcoming the seed-problem, and it seems that they represent a special case which is microspermous in all respects except the megaspermous characters of the large seed and the absence of herbaceous derivatives; and *Quercus*, when studied in south-east Asia, is seen to be a tropical product. The other three, however, are clearly most unusual and interesting examples of trees which have become mainly microspermous and temperate while retaining the old fruiting mechanisms (arillate in *Euonymus* and *Taxus*) and woody habit: *Euonymus* is also tropical, and *Aesculus* is near the tropical Sapindaceae.

8. *Herbaceous habit.* The massive fruits and seeds of the megaspermous trees cannot be produced on such juvenile shoots as those of herbs, e.g. Guttiferae, Lecythidaceae, Sapotaceae, Cupuliferae, without herbaceous forms. Consequently, the herbaceous habit could not have been evolved until the tropical tree-ancestor had attained the microspermous stage. Now it is commonly stated that in many large genera, as *Hibiscus*, *Cassia*, *Mimosa*, *Solanum*, *Veronica*, *Vernonia*, or *Dracaena*, there are all transitions from tree to herb. But, all these cases refer to more or less microspermous trees. When one considers megaspermous trees, I can find no genus (unless *Cassia*) and very few families in which transitional forms occur (Leguminosae, Rosaceae, Sterculiaceae, Apocynaceae, Euphorbiaceae, and Gramineae, for example).

There is, therefore, in fact an important difference between the typical megaspermous tree and the herb. There is a great gap between them which is bridged by the tropical microspermous tree. The transition from the tropical tree to the herb is a long and difficult process of the evolution of a microspermous fruit, contrary to all precedence, of which botany has not yet begun to take cognizance, cf. Guttiferae–Hypericaceae, Chrysobalanoideae–Rosoidae, Moraceae–Urticaceae, Bombacaceae–Malvaceae, Bignoniaceae–Scrophulariaceae, Bambusaceae–Gramineae. It is clear that neither herbaceous types as Ranales and Helobiae nor microspermous trees as *Casuarina* and *Populus* can be regarded as primitive, and the microspermous Amentiferae, as *Salix* and *Populus*, though curiously without truly herbaceous forms, appear fundamentally different from the megaspermous *Juglans*, *Quercus*, and *Fagus*. Likewise, such megaspermous families as Annonaceae, Myristicaceae, Burseraceae, Sapotaceae, and Palmaceae without herbaceous allies demand studies in physiological maturity for their satisfactory understanding. Indeed, it is

clear that a study of the physiological thresholds for flowering and fruiting in tropical trees should be undertaken and, being cognate with bud-grafting for early fruiting, it is a matter of practical importance (a search of tropical agricultural-horticultural literature may reveal some published information already). It should be borne in mind, too, that the leptocaul twig, necessary for the microspermous fruit, is essentially a precocious organ developed at the expense of previous assimilation and, thus far, resembles the seedling; and that the herb is also little more than a seedling matured at low threshold.

9. *Conclusion.* The immediate ancestors of modern flowering plants must have been sparingly and sympodially branched, soft-wooded, tropical trees of low or medium height, with massive twigs bearing spirally arranged compound leaves without distinct internodes, and reproduced by large arillate seeds borne on massive red follicles, succeeding terminal flowers or inflorescences. The more remote ancestors appear to have been monocarpic and monocaulous, with the Cycad-habit.

No such plants now exist, but many carry traces of this ancestry. The primitive form is shown, I think, in the habit of palms, pandans, tree-Senecios, tree-Lobeliaceae, tree-Euphorbias, tree-Paeonies, bottle-trees (*Adansonia*, *Brachychiton*), Cacti, *Carica*, Araliaceae, and so on, even to brussels sprouts, which owe their curious appearance to this primitive form: in fact, almost any 'funny tree' is funny because its form is primitive and, now, unusual. As a corollary, woody parenchyma appears, not as a modern feature as maintained by many wood-anatomists, but primitive in accordance with the habit of the early angiosperm (e.g. *Cecropia*, *Macaranga*, *Carica*, &c.).

EFFECT ON ANIMALS

In modern tropical forests, perhaps 50 per cent. of the trees and woody climbers have edible fruits. Of this portion, perhaps 90 per cent. have berries and drupes and only 10 per cent. have arillate seeds or pulpy seed-coats. But these 10 per cent. are certainly the most nutritious because the aril or pulpy testa is rich in oils, carotinoids, and other complex substances.

In the ancestral forests of early flowering plants, as the Durian theory implies, all the trees must have been hung with red lanterns of arillate black seeds, contrasting vividly with the green foliage, and there must have been much more food for animals in the trees. Imagine timeless forests consisting wholly of durians, instead of less than 1 per cent. as in Malaya now: imagine forests filled with red chestnuts and pulpy seeds: and the effect that the modernization of forests must have had on forest herbivores can be seen. In the beginning of the forests of flowering plants there was every inducement to climb, jump, and fly among the low stout branches after the fruits, and the roughly simultaneous origin, therefore, of flowering plants, birds, and mammals does not appear extraordinary. But, as the microspermous trees evolved and heightened and complicated the forests, there was less to eat both quantitatively and qualitatively. Modern monkeys, so isolatedly various, are only relics, as the fossil record is proving, of those which feasted in the early

forests. Mammoths grew extinct, no wonder, with herbs instead of durians to feed upon: and elephants, too, in the declining orchards. Parrots and squirrels, on the other hand, have the means of dealing with the modern nuts and seeds. Sloths hang on with leaves: monkeys become omnivorous: but fruit-eating birds and bats survive on the remaining arils and pulpy derivatives. These arils, so chemically rich, may have been an important speciating factor, for the converse effect can be seen in the poverty of variety of fruit-eating animals in the vast forests of oak, beech, pine, and hazel in the north temperate region.

Note. This heightening of the forest by the modern tree gave the Primate-environment. The development of the microspermous habit gave the optimum herbivorous environment, which brought the Primate back to ground.

EFFECT OF ANIMALS ON FRUITS

Armour. High pressure of animals leads to the eating of immature fruits and consequent waste of immature seeds. More loss is suffered to-day by tropical trees from attacks of squirrels, bats, honey-bears, and monkeys, which strip the immature fruits, than from insect-borers or disease. One can be sure that, from an early state in the evolution of flowering plants, the immature fruit must have been protected. There are three general means.

Firstly, there is camouflage by greenness among the foliage: that is to say, the reproductive mechanism of the flowering plant has two attractive stages, one for pollination and the other for seed-dispersal, and between them comes an inconspicuous stage. Large fruits, however, cannot be concealed, though the early arboreal vertebrates may have been short-sighted, and other means must be found.

Secondly, there is mechanical protection such as can be provided by a woody fruit-wall (which cannot become effective until the fruit has stopped enlarging), by persistent sepals, by a dense coating of hairs, and, particularly, by an armour of spines which can become effective by the hardening of the spine-tips as soon as the fruit begins to be conspicuous in size.

Thirdly, there is the chemical method of unpalatability by acidity, astringency, and poison. Nowadays this method is clearly the most effective, as it is the most universal: in fact, if they were not so protected, most modern fruits could not survive in the tropical forest.

There is much evidence, however, which suggests that the armour of spines has been an important and primitive factor. Just as the aril is comparatively rare among modern flowering plants, so are spiny fruits: and the association of spines and arils is so close as to imply that the armour of the durian is as characteristic as its aril. Where spiny fruits occur, arils may be expected in the same genus or in related genera and vice versa.

Examples: *Victoria*, *Rheedia* (Guttiferae), *Sloanea* spp. (Tiliaceae and other non-arillate genera), *Cnestis* (Connaraceae): *Nephelium*, *Xerospermum*, *Paullinia* (sect. *Castanella*), *Schleichera*, *Lepisanthes* (Sapindaceae, with the non-arillate *Paranephelium* of Malaya resembling a small durian); *Aglaiia* with rudimentary spines (Meliaceae, but *Flindersia* with muriculate fruits and winged seeds as the transition

between the durian-type and *Swietenia*); *Sindora* (Leguminosae): *Carpotroche*, *Mayna* (Flacourtiaceae); *Rinorea*, *Alsodeia* (Violaceae, ? non-arillate); *Monordica*, *Cucumis*, *Sechium*, and *Echinocystis* (Cucurbitaceae, with pseudo-arillate seeds); *Tabernaemontana* spp. (Apocynaceae); *Ricinus*, *Mallotus* (Euphorbiaceae): *Geanthus*, *Amomum*, *Globba* (Zingiberaceae): *Canna*.

The presence of spines on the fruits of *Allamanda* (Apocynaceae), *Datura*, some Bignoniaceae, *Melastoma*, *Galium*, *Ranunculus*, and *Dichaea* (Orchida-

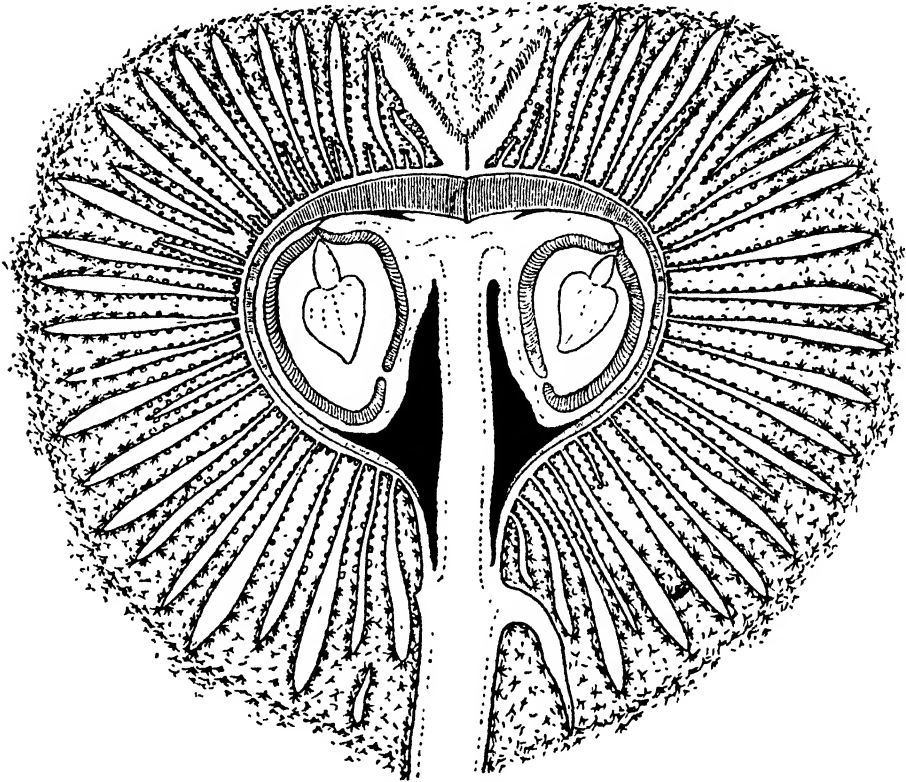


FIG. 25. The ripe, as yet indehiscid, fruit of *Mallotus barbatus* (Euphorb.) in longitudinal section, showing the rather stiff, irritant, stellate-hairy, spines and the seeds with pulpy sarcotesta (outer integument), hard palisade layer of the inner integument, and a rudimentary aril on the micropylar side of the funicle: illustrating the complexity of spinous fruits ($\times 6$).

ceae) are, therefore, suggestive as relics, exactly as with *Bixa*, which has a rudimentary aril and pulpy testa.

The absence of spines from the Dilleniaceae and their development of the persistent sepals instead, suggests that this family may have had from a very early stage a different mechanism for protecting the arillate fruits.

Now, as far as I have discovered, the spines on fruits are definite outgrowths developing immediately under the primary and larger peltate or glandular hairs of the ovary (glandular hairs being juvenile modifications of the peltate). Peltate scales are a Pteridophyte-feature, and thus spininess is related with yet another archaic character. Spininess, indeed, typifies the petioles of many tree-ferns (*Cyathea*).

The imbricating, backward-pointing scales of the Lepidocaryoid palms develop from the peltate scales of the young ovary and appear as the modern

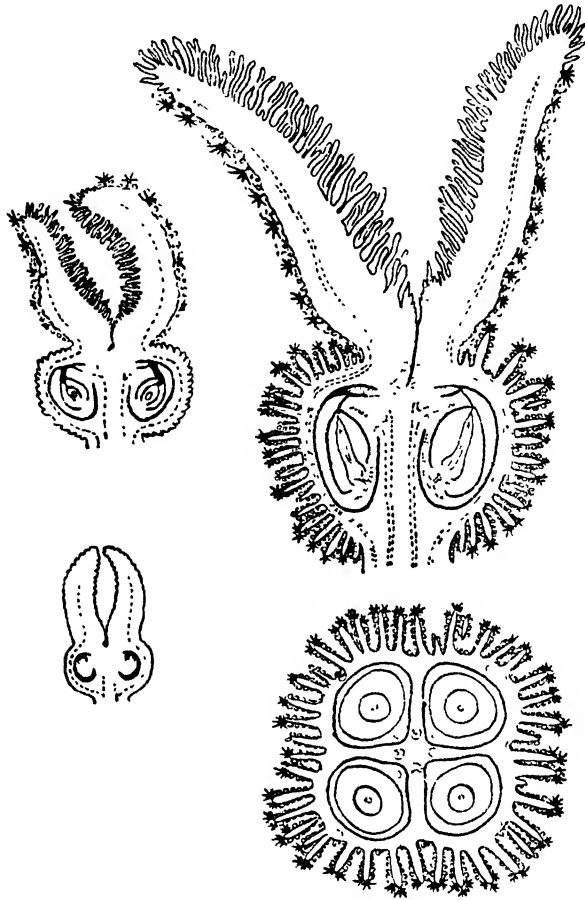


FIG. 26. Developing flowers of *Mallotus barbatus* (Euphorb.) to show the early origin of the spines of the ovary beneath stellate hairs, the basipetal development of the styles and ovary, and the oleaginous conducting tissue (dotted) of the stigmas leading to the aril-flap over the micropyle (in the right-hand mature flower): the spines of the ovary with both glandular hairs (on their stalks) and stellate hairs (at their tips) ($\times 15$).

armour of their modern drupe, adapted from the spiny arillate capsule of the proto-Palmaceae.

The spines of *Annona muricata* and *Rollinia* (Annonaceae) appear in the same light, as transferred to the carpel-points. In the case of *Artocarpus*, the function of the aril is transferred to the ovary-wall and that of the spines to the perianths of the minute flowers, thus developing a durian-fruit from an inflorescence. *Pandanus* is comparable. In the case of *Castanea*, the protective function has been transferred to the spiny bracts, and the Spanish-chestnuts

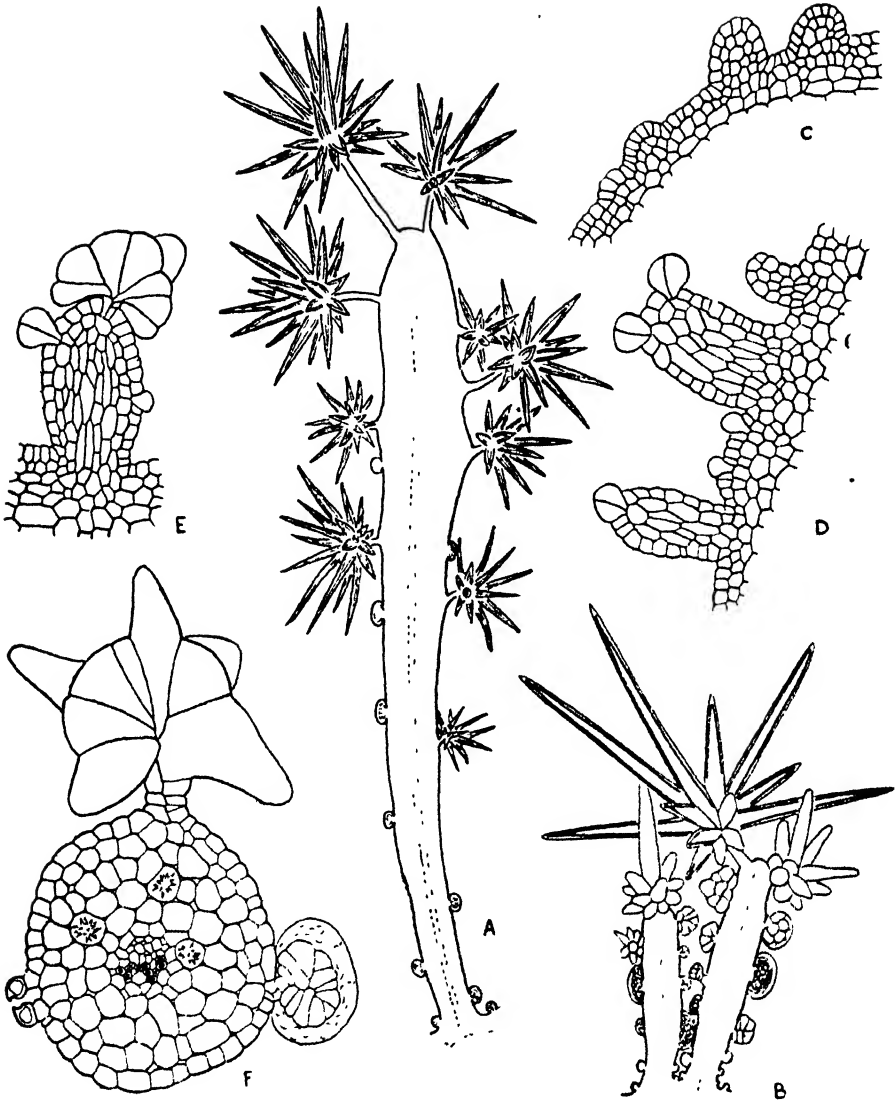


FIG. 27. The spines of the ovary (B) and fruit (A) of *Mallotus barbatus* (Euphorb.), showing the stellate hairs at the apex and upper part of the spine and the glandular sessile hairs in the lower part (developed basipetally after fertilization) (A $\times 25$; B $\times 40$). C-E, stages in the basipetal growth of the spines on the ovary, developing stellate hairs at their apices ($\times 225$). F, a transverse section of a spine of a half-grown fruit, showing a young stellate hair, a glandular hair, a stoma, and the central vascular bundle ($\times 225$).

appear as Horse-chestnuts in the second degree, from inflorescences instead of individual flowers. Certainly *Artocarpus* and *Castanea*, when correctly interpreted, will prove to be the key-genera to the evolution of the Moraceae–Urticaceae and the Cupuliferae, just as *Durio* is to the Bombacaceae–Malvaceae.

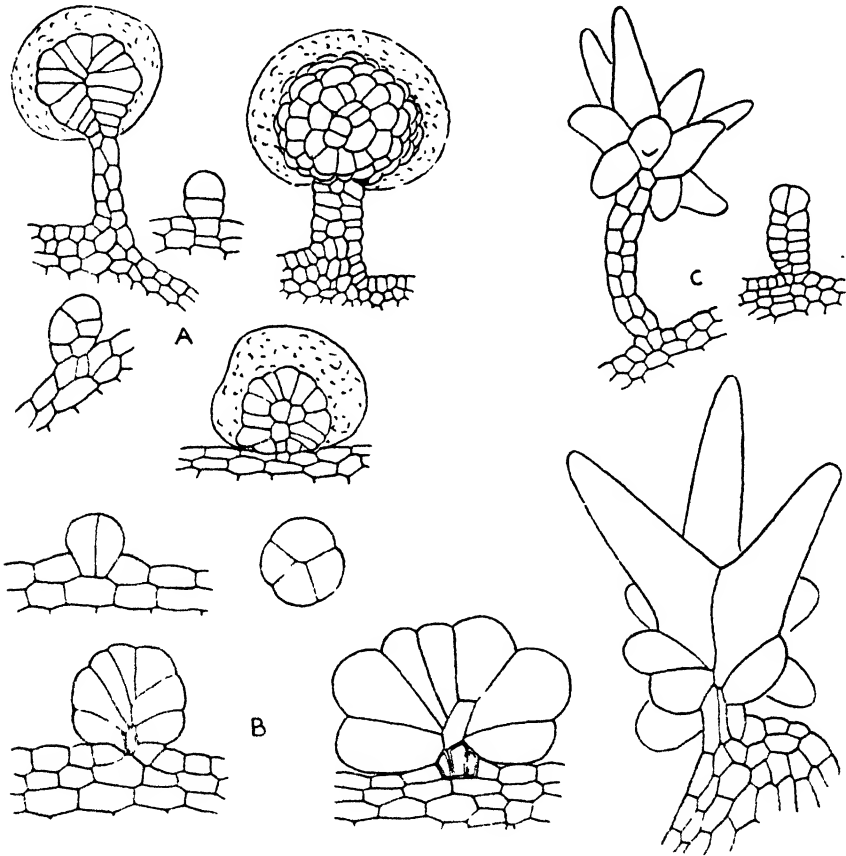


FIG. 28. Developing hairs on the spines of *Mallotus barbatus* (Euphorb.), showing how stellate, glandular, and, by simplification, all other hairs can be derived from peltate hairs initiated by 4 longitudinal cell-walls in an enlarged epidermal cell. A, sessile and stalked glandular hairs ($\times 225$); B, sessile and sub-sessile, young stellate hairs ($\times 400$); C, stalked, young, stellate hairs ($\times 225$).

In its arillate spiny capsule, large seeds, and peltate scales *Durio* appears as the most typical modern representative of the primitive angiosperm-fruit. *Durio* is modern, however, in its tree-habit with slender twigs and sprays of simple leaves: hence, its ramiflory. In contrast, the well-known Horse-chestnut (*Aesculus*) appears as the best temperate outlier of the tropical proto-angiosperms. Its massive, spiny capsules with large, but non-arillate, seeds are terminal on massive shoots with compound leaves, the massiveness of the shoot (well known in botanical classes because of the large bud) preventing

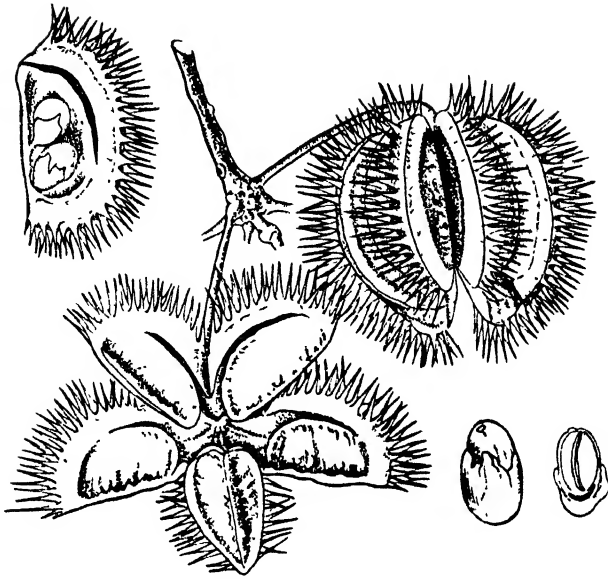


FIG. 29.

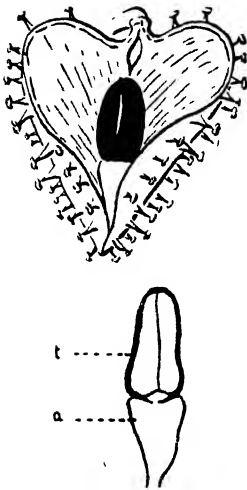


FIG. 30.



FIG. 31.

FIG. 29. The spinous fruit of *Sloanea hongkongensis* (Tiliaceae—Elaeocarp.), with arillate seed ($\times \frac{1}{2}$) (from Hooker's Ic. 11. No. 2628, Ser. IV, vol. vii, pt. ii, 1900).

FIG. 30. The spiny 1-seeded legume of *Sindora* sp. (Caesalp.), with black testa (*t*) and red arillode (*a*), the spines resin-tipped ($\times \frac{1}{2}$).

FIG. 31. The dehiscent, spirally coiled, brownish legume of *Acacia auriculaeformis*, with black seeds dropping down on yellow waxy funicles ($\times 1$).

ramiflory. *Aesculus* is a key-genus to the Sapindaceae–Aceraceae, and shows the utmost geographical possibilities of the mechanism of the primitive angiosperm.

To the attributes of the primitive angiosperm there must, therefore, be added peltate scales and spiny fruits, and both characters open further vistas of inquiry among the families of modern flowering plants, e.g. the stellate hairs as relics of peltate scales (as in *Solanum*, where the hairs help to distinguish modern, glabrous or simply hairy, slender species as *S. dulcamara*, *S. nigrum*, and *S. parasiticum* from massive *Carica*-like species as *S. quitoense*

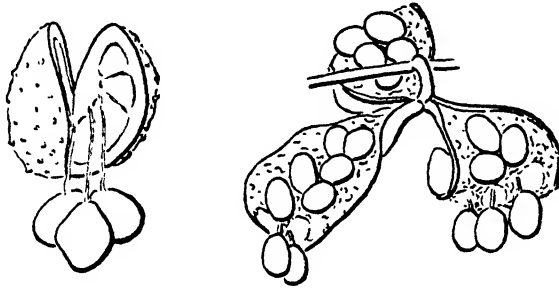


FIG. 32. A dehiscent follicle of *Michelia champaca* (left, Magnoliaceae) with pink seeds (sarcotesta) and the dorsally dehiscent follicles of *Xylopia fusca* (right, Annonaceae) with bluish testa (sarcotesta), neither with aril but both with pseudo-funcles (see text) ($\times \frac{1}{2}$).

and *S. grandiflorum* with dense coatings of stellate hairs), and the hydathodes of Bromeliaceae.

Dangling seeds. Only the small-fruited *Durio Griffithii* shows the hanging arillate seed which is so conspicuous in *Sloanea*, Meliaceae, and Leguminosae, or in *Sterculia*, *Gloriosa*, and *Erythrina* which lack the aril. Nevertheless, dangling is a character of the arillate or pseudo-arillate seed as shown also by the following four examples:

- a. Magnoliaceae. There is no aril, the pink or red testa being pulpy; and there is practically no funicle (as in Annonaceae), but the seeds hang down on slender threads which are the uncoiled spiral thickenings of the protoxylem-vessels of the raphe.
- b. *Xylopia fusca* (Annonaceae). The blue-grey seed has a pulpy testa but no aril: it hangs on a stalk which is the vascular bundle of the placenta after the friable pink endocarp has broken away from it in the open fruit.
- c. *Guioa* (Sapindaceae). The seeds are arillate but sessile: when the fruit opens they drop out and hang on a long thread developed from the aril at the micropyle.
- d. *Gyrotarpus* (Thymelaeaceae). The seeds appear to have a very astonishing brown furry aril (I have not been able to examine its morphological character) and, when the dry capsule opens, the one or two seeds, which it contains, drop down on slender threads which strip from the septum and dangle like spiders.

When one considers also that seeds, more or less arillate, hanging on funicles 1-4 cm. long, are characteristic of the huge genus *Acacia* and that the large, more or less arillate seeds of the big genus *Swartzia* (Leguminosae)

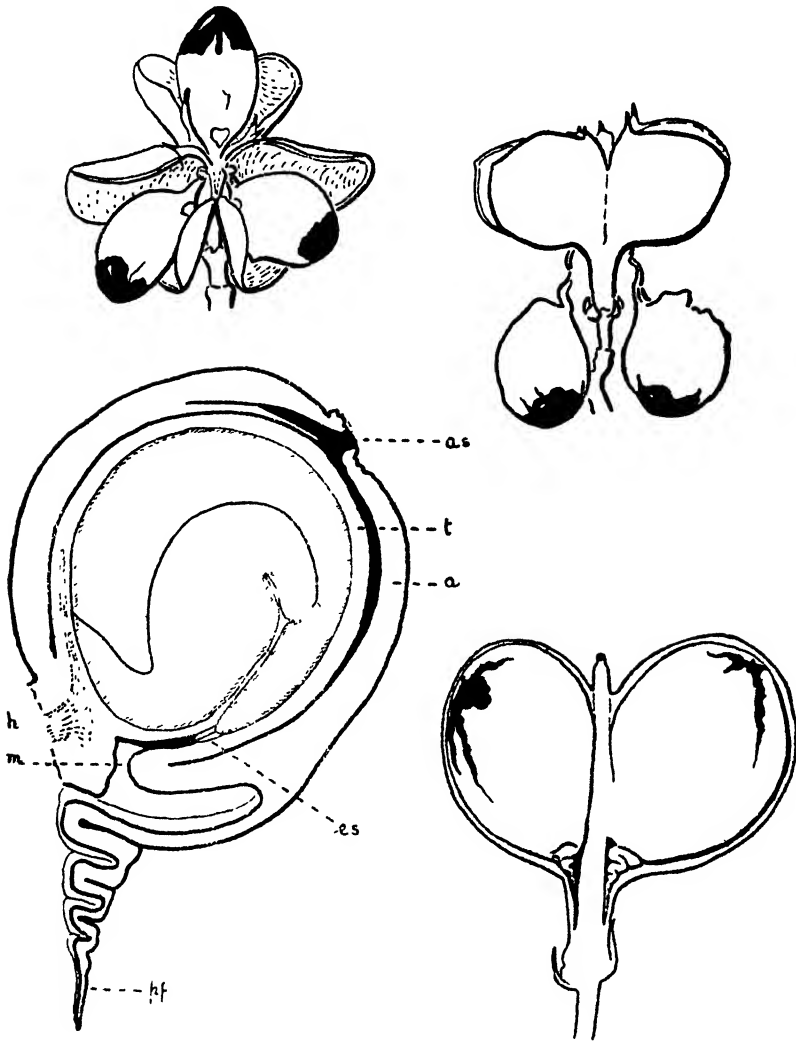


FIG. 33 Fruits and seed of *Guaoa pubescens* (Sapindaceae), with black seeds and yellow aril developing a pseudo-funicle (*pf*) from its micropylar outgrowth. (The two upper fruits $\times 2$; the section of the unopened fruit $\times 3$; the longitudinal section of the seed $\times 7$.) *a*, aril; *as*, arilostome; *es*, endostome of inner integument; *h*, hilum; *m*, micropyle; *pf*, pseudo-funicle; *t*, testa.

behave in the same way (having even longer funicles in some cases), it is clear from all these varied examples that dangling edible seeds must have a biological significance. As movement helps the unsophisticated vision it seems that they catch the eye of birds which peck off the pulp. Possibly, in the

dry Australian forest, the waxy aril of the small seeds of *Acacia* supply considerable food and, even, liquid to the birds to which they may appear as spiders or caterpillars on threads. Certainly a dangling seed, pecked and

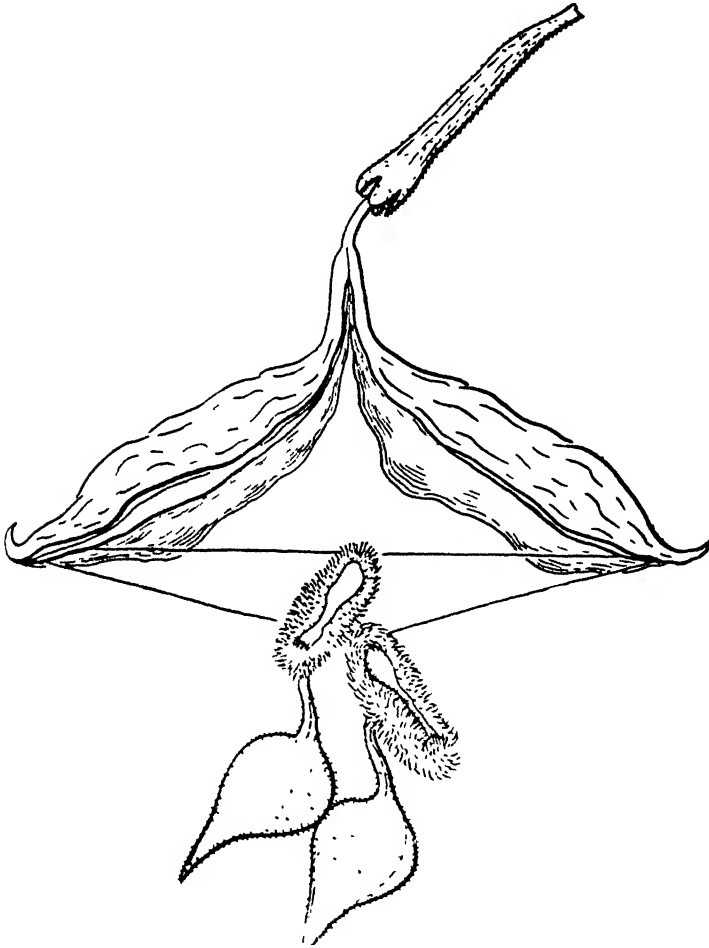


FIG. 34. The dehiscent, two-valved, dry, brownish capsule of *Gyrinopsis* sp. (aff. *G. Cumingiana*, Corner, Singapore Field, No. 29195: Thymelaeaceae), showing the two seeds (one from each loculus) with brown furry appendages (? arils) and pseudo-funcles derived from the split central portion of the septum on dehiscence ($\times 2$).

dropped, has more chance of survival than a durian-seed which may be crushed before being swallowed, though one such seed indigested will start with a favourable supply of manure.

So another feature of the flowering plant, namely, the length of the funicle, gains importance. Why should seeds have funicles? Why do the Acanthaceae have specially modified funicles? The only answer, or thought, that can yet be supplied is that they possess these features by inheritance from the

primitive arillate fruit, just as such funicles are retained as useless relics in the indehiscent pods of *Parkia* and *Cassia*. In fact, the problem is, rather, how seeds came to be detached from their funicles.

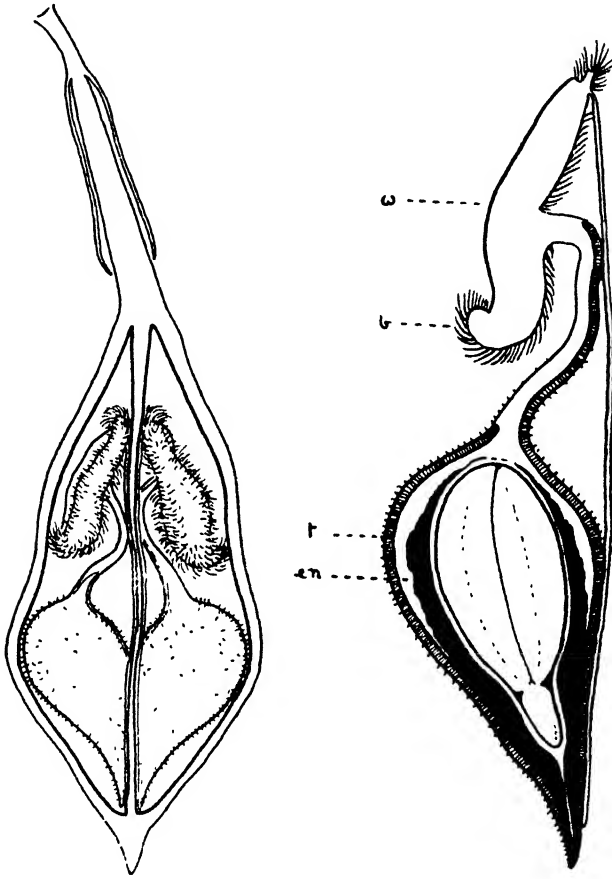


FIG. 35. A full-grown capsule in section ($\times 2$) and a mature seed in section ($\times 4$) of *Gyrinopsis* sp. (Corner 29195, Thymelaeaceae), showing the blackish, carbonaceous, shortly hairy, bomb-like body of the seed with rigid, curved neck and brown hairy appendage with white disc (*w*): the pseudo-funicle, joining the appendage to the bomb of the seed, being part of the septum of the capsule and splitting longitudinally into two threads on dehiscence of the capsule. Note how the embryo is suspended in the bomb of the seed with an air-cushion (black) around it. *b*, brown hairs of the appendage; *en*, the remains of the inner part of the testa; *t*, the black carbonaceous layer of the testa; *w*, the smooth white disk of the appendage.

Colour. No more vivid contrast can be imagined than a red fruit with hanging black seeds and scarlet arils against green foliage. The very blackness of the seed emphasizes the aril. Modifications into yellow fruits and yellow arils seem not to affect the result. But the problem raises the question of the colour-blindness—whatever that may mean—of animals. In my own experience, I have found that red is so appealing to the Coco-nut Monkey (*Macaca*

nemestrina) that, even when too sick to stand or eat or swallow, yet he will start with joy at the sight of a red fruit. I consider it to be no coincidence that bird-flowers are red, that parrots are red, that nutmegs have red mace or pulp, that the primitive angiosperm fruit should appear to have been red, that savages should paint their faces, monkeys their buttocks, and women their nails red, that holly-berries and red crackers should decorate Christmas, that flags should be red, that warning signals should be red, and 'On the whole it seems that bony fishes are more attracted by the red end of the spectrum than by the violet end. This accords with the evidence of anglers who find red baits very effective' (Pincher, 1947). What is redness to attract and gladden life?

Smell. The final characteristic of the durian is its smell. An even stronger smell is that of the Chempedak (*Artocarpus integer*), which has modified durian-fruits, or the Horse-mango (*Mangifera foetida*). Ducke records a similar smell for a species of *Swartzia* (Leguminosae), the pods of which are eaten by tapirs and pigs in Brazil, as durians are in Malaya. There is certainly more to be learnt about the significance of this factor as the ultimate appetizer, as there is with the chemical protection of the immature fruit. There must have been many more smells to attract the early elephants and tapirs and the short-sighted beasts; and it would seem that to them we owe the selection and survival of the durian.

RECAPITULATION

As any of the foregoing passages could furnish material for a book I will recapitulate for the sake of clarity, in the sequence in which I believe their evolution has generally occurred, the main steps in the development of the modern tree, bearing in mind that it is the fruit and seed, as the dispersal mechanism, which are the more significant reproductive parts of the flowering plant.

A. *Pachycaul Stage*

Stems massive, sappy, soft-wooded, not or sparingly branched, with little or no internodal development: megaphyllous: megaspermous.

1. *Monocaul or Cycad phase.* Low, stout, unbranched monocarpic trees without internodes, with peltate scales; leaves multipinnate, probably with a spiny rachis (flowers primitively terminal, gigantic, uncondensed, of pinnate stamens, and large, peltate-scaly carpels: fruit as a cluster of large spiny follicles, perhaps 0.5–1 m. long, ripening red and dehiscing with many large black seeds, perhaps 2 cm. long, covered with a red aril and hanging on persistent funicles).

This phase is largely hypothetical. It may have been truly monocaulous and monocarpic or sympodial, polycarpic, and pseudomonocaulous as *Cycas* itself: the resemblance to the tree-fern suggests, however, lack of branching in the first place. As a tree-form, this phase persists not only in Cycads but, with some modification in the development of internodes, in palms and the

saplings of many dicotyledonous trees with compound leaves (*Carica*, *Cecropia*, *Schizolobium*, *Bombax*, *Aralia*, &c.), the sapling stage recapitulating, in fact, this ancestral phase in the evolutionary history of modern trees.

Reduction of the leaf to an entire blade introduces most of the remainder of modern saplings and, as an instructive instance, the monocarpic *Agave* which, having become microspermous, shows that the massive rosette plant, without internodes, may have originated directly from the Cycad-form of tree. At least, *Agave* indicates the necessity for re-examining the massive growth-forms of tropical and subtropical Monocotyledons.

The primitive spiny fruiting carpel clearly represents the pinnate megasporophyll reduced to a bud-scale or basipetal phyllode, and is thus homologous with the spiny petioles of *Cyathea* and *Cycas*. But, in the evolution of the angiosperm, the full development of the megasporophyll or carpel is postponed until after fertilization. This early basipetal or phyllodic tendency not only continued to characterize the monocotyledonous leaf but the flower in general, leading eventually to the gamophyllous floral whorls and the inferior ovary. (The most extreme reduction is seen in *Welwitschia*.)

2. *Monocotyledonous phase*. Suckering must have entered sooner or later in the Cycad-phase, as a result of excess photosynthesis in the monocarpic tree, especially without secondary thickening. Thus in the palms, Gramineae and Scitamineae, tufted tree-forms have arisen (*Metroxylon*, *Bambusa*, *Ravenala*, *Musa*) and, with microsperry and internodes, these forms have produced the characteristically tufted monocotyledonous herbs as the grasses, sedges, Zingiberaceae, and Marantaceae, in which the rhizome is a secondary development of the sucker. Such herbaceous forms do not occur in Dicotyledons where the herb has evolved from trees with secondary thickening which obviates suckering, and where the seedling does not go through the stage of the *Agave*-rosette without internodes.

Ravenala is usually mistaken as a genus with two species, *R. guyanensis* and *R. madagascariensis*, evidencing discontinuous distribution. Actually they are generically distinct. *R. madagascariensis* has the lateral inflorescences, flowers, seeds, and pollen of the South African *Strelitzia*, and *R. guyanensis* has those characters which distinguish the South American *Heliconia*. Thus, geographically, the two natural groups are coherent and, phyletically, they indicate the ancient and relic character of this tree-form.

3. *Carica-phase*, resembling the Cycad-phase but with incipient internodes and sparse branching of the less massive stem: in Dicotyledons, not suckering owing to the elongation of the seedling and the presence of secondary thickening.

Typical of this form is *Pandanus*, as well as *Carica*: perhaps, also, *Hyphaene*, the Cactaceae and Nymphaeaceae (as aquatic sub-herbaceous derivatives). But, at the same level of tree-form, though with more or less hard-wooded and much less massive trunks, occur also many tropical trees scattered among such families as Simarubaceae (*Eurycoma*), Campanulaceae, Solanaceae, Compositae, Bignoniaceae (*Oroxylon*, *Pajanelia*), Araliaceae, &c. It is impossible to draw a sharp line between them and the following two stages, cf.

Artocarpus, *Cecropia*, *Macaranga*, but it is interesting to observe that they may also give rise to herbaceous derivatives through introduction of micro-spermy before the true modern tree-form has been reached. *Cecropia* and *Macaranga*, it may be noted, with their low height are restricted mainly to secondary forest or priseres of the tropics, thus evidencing the ecological evolution of the tree-form.

The chief variation in this phase seems to be the position of the inflorescence, whether terminal (Bignoniaceae, *Pandanus*) or lateral (*Carica*, *Nymphaea*, as *Cecropia*, *Macaranga*, and the Araliaceae).

The bottle trees, as *Adansonia* and *Brachychiton*, appear to come between the *Carica*-phase and the *Dysoxylon*-phase.

B. *Leptocaul Stage*

Twigs more or less slender, much branched, more or less hard-wooded: with internodes more or less well developed: megaphyllous or microphyllous: megaspermous or microspermous.

Megaspermous

4. *Dysoxylon*-phase. *Dysoxylon* (Meliaceae) is typical of this important kind of tropical tree with megaphyllous compound foliage, spirally or decussately arranged on rather massive ascending twigs. *Dysoxylon*, also, retains characteristically the arillate fruit as a fleshy loculicidal capsule. Many Leguminosae, Sapindaceae, Burseraceae, Anacardiaceae, Bombacaceae, Sterculiaceae, &c., belong here, as well as *Bocconia*, *Hevea*, and *Artocarpus* pr.p.

Again, both lateral and terminal inflorescences occur, as in the *Carica*-phase, but, as in the following two groups, there are no herbaceous derivatives, this being the megaspermous tropical tree *par excellence*.

5. *Magnolia*-phase. This resembles the preceding phase but has simple leaves, representing either terminal leaflets (*Mangifera*, *Lucuma*, *Persea*) or webbed compound leaves (*Dillenia*, and, perhaps, *Magnolia*), and less massive twigs. Typical are Magnoliaceae, Dilleniaceae, Sapotaceae, *Barringtonia*, *Persea*, *Quercus*, &c., but there are many transitions to the foregoing (as in Sterculiaceae) or the following (as in Myrtaceae, Melastomaceae, and Elaeocarpaceae). The pagoda-trees of *Terminalia*, *Achras*, *Palaquium*, *Elaeocarpus*, *Sloanea*, and *Cerbera* are characteristic of this phase (see Corner, 1940). The leaves may be large or small.

6. *Myristica*-phase. This agrees with the *Magnolia*-phase in the simple leaves, but they are arranged alternately or decussately in one plane to give the applanate foliage, or horizontal spray, as the most advanced foliage-display of trees, and the twigs are, typically, slender. The trees are usually more or less microphyllous. Characteristic are Myristicaceae, Annonaceae, and many modern genera of tropical and temperate trees as *Fagus*, *Carpinus*, *Durio*, *Symplocos*, pr.p., *Diospyros*, *Lecythis*, *Memecylon*, *Eugenia*, pr.p., &c.

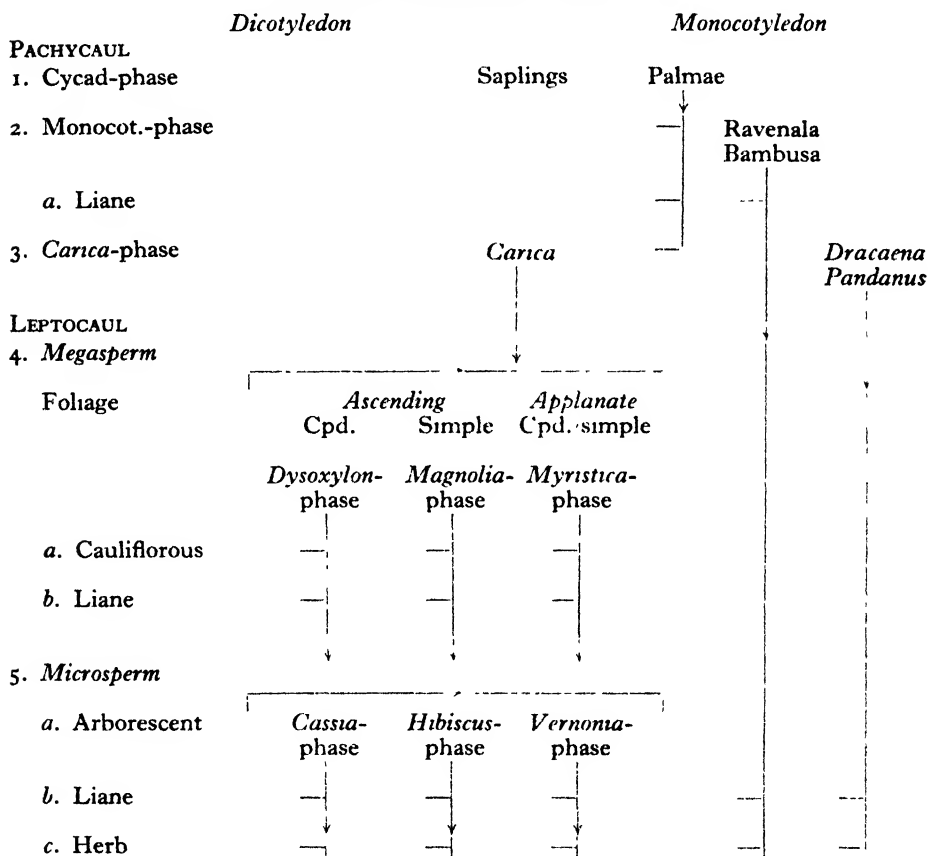
7. *Cauliflorous* phase. This phase may be imposed on any of the three preceding phases with slender twigs and massive flowers or fruits, but is more

characteristic in the *Myristica*-phase, cf. *Myristica*, *Polyalthia*, *Diospyros*, *Eugenia*, *Durio*, &c.

Microspermous

This most modern kind of tree may repeat any of the preceding phases 4, 5, and 6, which may be represented typically by *Cassia*, *Hibiscus*, and *Vernonia*, all of which give, characteristically, herbaceous derivatives, though there are examples as *Weinmannia*, *Populus*, and *Salix* which are entirely woody.

Diagram of Angiosperm Tree-forms



When the theory is represented diagrammatically, one can see:

a. That the gap between the Cycad-phase and the Leptocaul is filled largely by Monocotyledons which may be taken as representing the proto-Dicotyledons, just as Conifers and Gnetales are modern representatives of the protogymnosperms. While the Dicotyledons have produced the succeeding stages in forest-evolution, the monocotyledonous tree-habit seems to have represented the early ones. Thus, the tropical tree-fern, pteridosperm-, and cycad-forests seem to have given place to palm-forests before the broad-leaved

dicotyledonous forests were established, and the absence of normal secondary thickening in Monocotyledons, as well as the absence of a tap-root and internodal development in the seedling, may be a primitive tree-fern character. The problem may, in fact, be not why the Monocotyledons diverged from the Dicotyledons, but why the dicotyledonous seedling diverged from the Monocotyledons.

b. That there is a strong difference between the woody and herbaceous habits in the first group of dicotyledonous trees (*Leptocaul-megasperm*), but not in the second (any more than in the two monocotyledonous series giving herbaceous derivatives). Failure to distinguish these two classes of tree is the cause of the confusion existing at present in attempts to classify Dicotyledons into woody and herbaceous groups. The *Meliaceae* and *Sapotaceae* are not equivalent to the woody *Malvaceae* and *Rubiaceae*.

c. That the herbaceous form has been derived along two main lines, from the pachycaul and from the microspermous leptocaul (which may or may not be secondarily soft-wooded), and that this analysis will help to explain some of the misunderstandings which exist, also, in reference to the herbaceous habit.

d. That the liane is a special case derivative from all phases of the leptocaul tree (as well as from the Monocotyledon).

e. That all the phases are well and fully represented by tropical plants, but only the microspermous and the monocotyledonous herbs are well represented in the temperate floras, thus illustrating the conviction, which most botanists studying tropical forests must have formed, that no flowering plant could have emerged from the tropical rain-forests to the monsoon and temperate regions until it was fit, structurally and physiologically, to meet the rigorous conditions.

f. That (i) the microspermous and microphyllous arborescent *Casuarinaceae*, *Salicaceae*, *Cunoniaceae*, &c., and (ii) the megaspermous but temperate *Quercus*, *Fagus*, *Juglans*, *Aesculus*, &c., stand out as blind alleys in evolution, and require special consideration.

g. That the early angiosperms, being soft-wooded with large, compound, mesophytic leaves and large, non-dormant seeds, are unlikely to have left fossil evidence. The *Rhizophora* seedling, germinating *in situ*, may be the modern representation of an archaic, estuarine, proto-angiosperm habit.

h. That reduction in size, after a zenith in an optimum locality, always takes place, cf. *Lepidodendron* and *Calamites*; so the forests of the tropics are giving place to pasture through human interference, e.g. bamboo to grass.

i. That there is an enormous field of research opened to analyse the relative position of every family and genus of angiosperm in this evolutionary diagram. Every small specific group of tropical plants will need renewed investigation, for the majority cannot be placed by consulting published information, cf. the confusion over *Ravenala*.

CONCLUSION

I have avoided nearly all reference to the flower. As recently as 1930 it was written that 'the fruit contributes little or no evidence of value concerning

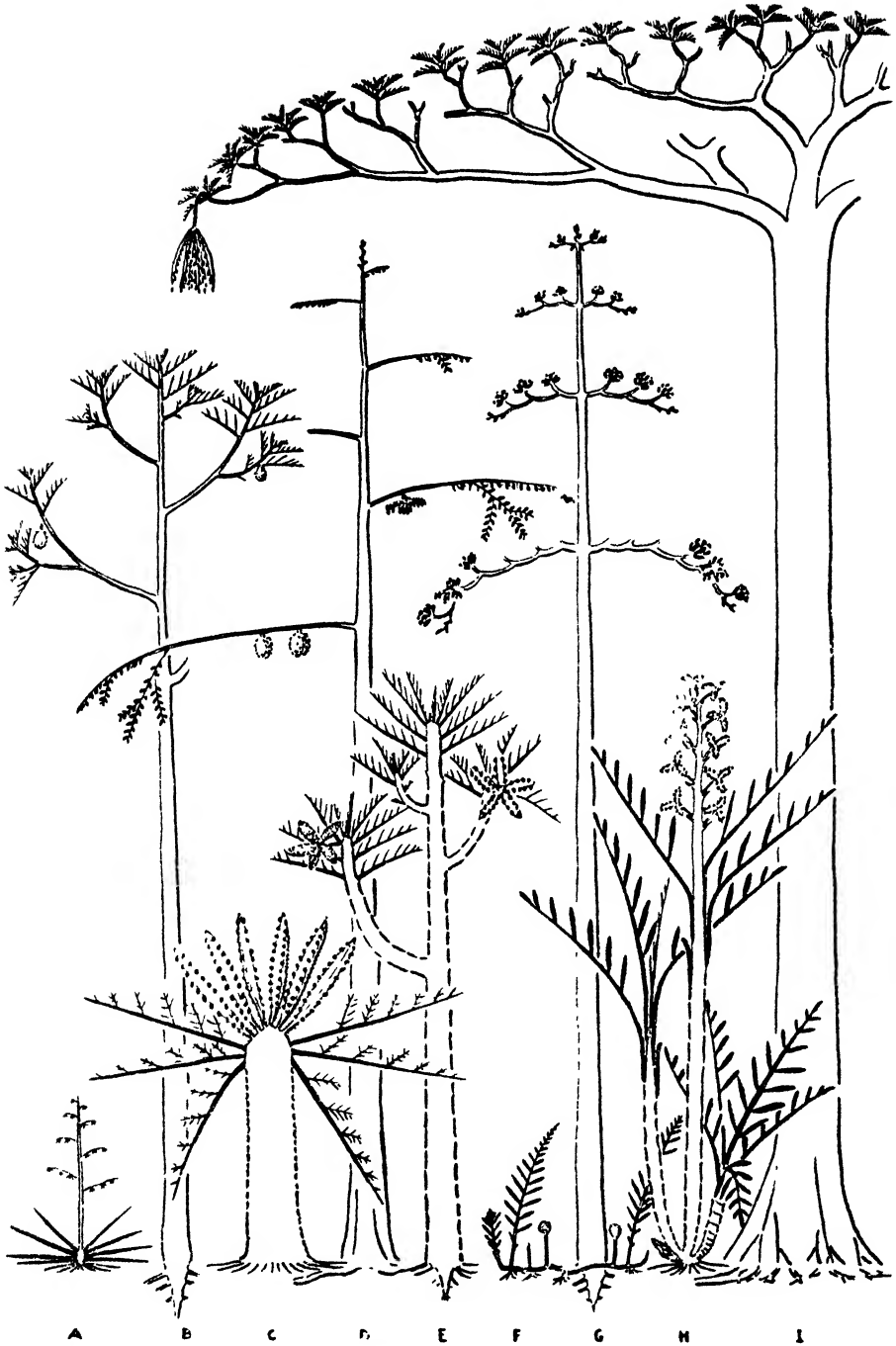


FIG. 36. Diagrams of the main tree-forms and of two sub-herbaceous derivatives (A, F). A, the *Agave*-rosette, as a direct reduction-form of C; B, the megaspermous leptocaul tree with spirally arranged pinnate leaves, giving ascending foliage; C, the hypothetical Cycad-form, monocaulous and monocarpic with a terminal cluster of spinous follicles with arillate

the primitive habit of Angiosperms'. The Durian-theory, as I have been advised to call it by Dr. H. Godwin, F.R.S. (Cambridge Botany School), shows that it is possible, from the study of tropical fruits, to arrive at a far-reaching understanding of the evolution of the modern tree and, perhaps, of most living accompaniments to the origin and dispersal of flowering plants.

The theory shows that from mesophytic Cycad-like trees, possibly monocarpic, with terminal flowers or inflorescences and large red spiny follicles disclosing black seeds with red arils, the tropical forests have gradually evolved the modern microspermous tree, as the antecedent to the dicotyledonous herb, and in so doing have both heightened the forest, thereby increasing its complexity particularly with regard to climbers and epiphytes, and also reduced its food-content for animal life. The theory directs attention to one of the most neglected aspects of biology, namely, the real life of the tropical forests, not only with regard to the dependence of the animals upon the plants, but particularly with regard to the enormous variety of tree-forms representing all sorts of combinations of evolutionary processes in varying response to the main trend. Thus the cauliflorous tree, the palm, and the pagoda-tree, as well as the berry, the drupe, the nut, and the size of the seed, find a natural explanation; *Aesculus* and *Artocarpus*, *Castanea* and *Pandanus* fall into place with *Sloanea* and *Durio*. But any hypothesis, shedding light on so large a field, must be continually readjusted as its new aspects are seen: and every case of genus or family will need its own particular consideration. Tree-habit, wood, leaf, bud, flower, fruit, seed, and root all supply independent criteria which must be analysed and estimated.

Now, the antecedents of *Archidendron*, *Delonix*, *Durio*, *Sterculia*, *Sloanea*, *Dysoxylon*, and so on, which had massive twigs of compound leaves, large flowers, and red arillate fruits, clearly represented a climax of tree-form in the optimum conditions for terrestrial plants in the tropical rain-belt, and were, thus, an expression of Xerophyton, or the land-plant. But the culmination of plant-growth on land, expressed in size and survival, is shown now not only by these very genera but by such families as Papilionaceae, Diptercarpaceae, Anacardiaceae, Lecythidaceae, Cupuliferae, and so on, which tend to a reduction in size and complexity of the twig, leaf, flower, and fruit, though retaining the big seed, as the forest-factor. It is these modern families of megaspermous tropical trees that I consider to represent, in their homoplastic diversity, the idea of Xerophyton. They are the modern tropical forests,

seeds, D, the cauliflorous tree with simple alternate leaves giving applanate foliage, leptocaul (cf. *Durio*); F, the *Carica*-form, pachycaul, with few branches, compound leaves, incipient internodes and arillate follicles (axillary); F, the cauliflorous Monocotyledon, as a rhizomatous sub-herbaceous plant, with aerial sprays of applanate, simple foliage, analogous with the cauliflorous dicotyledonous tree, but derivative from H (cf. Zingiberaceae); G, the pagoda-tree with arillate capsules (cf. *Sloanea*) and simple leaves in rosettes; H, the monocotyledonous pachycaul with suckers, monocarpic with arillate, spinous follicles and compound leaves (cf. *Ravenala*); I, the megaspermous leptocaul ancestor of Leguminosae with dehiscent arillate legumes and spirally arranged, compound leaves (cf. *Parkia*). (The picture needs primitive birds and mammals for completion.)

from which most of the temperate floras have been derived. They will last, apart from human interference, so long as the climate of the rain-forests persists, but, as this deteriorates, they will give place to the microspermous trees and, eventually, savannahs, as the golden age will always pass.

SUMMARY

Taking the Durian (*Durio zibethinus*) as the type of fruit, and using the Leguminosae, particularly, for exemplification, as well as *Carica*, *Artocarpus*, *Aesculus*, and *Castanea*, it is argued that the primitive angiosperm fruit must have been a red fleshy follicle, probably spiny, with large black seeds hanging on persistent funicles and covered with a red aril.

From this precept, it is argued further that the primitive angiosperm must have been a mesophytic, tropical, Cycad-like monocaulous tree with large pinnate leaves and peltate scales, probably monocarpic, and producing a terminal cluster of large arillate follicles.

Ramification with consequent reduction in size and complexity of the branches, leaves, flowers, and fruits, and the evolution of axillary inflorescences, have led to the modern tree-form with many slender twigs, simple leaves in horizontal sprays, small flowers, and greatly increased height.

Among modern tropical rain-forest trees a distinction is drawn between the more primitive *pachycaul* trees with massive unbranched, sparingly branched, or suckered (monocotyledonous) trunk, soft wood, and large leaves, and the orthodox *leptocaul* trees with relatively slender twigs and hard wood. A further distinction is made among *leptocaul* trees between the *megaspermous* and non-herbaceous and the *microspermous* from which the dicotyledonous herbaceous plants have been derived: transitions between these two kinds of trees appear to be rare. Cauliflory is a condition forced upon *leptocaul* trees with, usually, applanate foliage by the retention of the old massive forms of flower and fruit.

The principles of *axial conformity* (or the correspondence in size and complexity between appendages and the parent axis) and *diminution on ramification* are indicated as fundamental to the construction of land-plants.

Modern fruits as capsules, nuts, winged indehiscent fruits, and so on have been evolved from the primitive arillate fruit with consequent great loss in food-supply to forest birds and mammals.

The significance of the spiny armour, the colour, the dangling seeds, and the smell of the arillate fruit is discussed.

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Leaf Analysis and the Nutrition of the Oil Palm (*Elaeis guineensis* Jacq.)

BY

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AND

H. M. GRAY

With five Figures in the Text

INTRODUCTION

THE theory of leaf analysis in relation to the mineral nutrition of plants has recently been reviewed (Goodall and Gregory, 1947; Lundegardh, 1947) and will not be considered here, except in direct relation to the experimental results. The work described in the present paper is an extension of earlier work on the nutrition of the rubber tree in Malaya (Chapman, 1941). It was begun in 1938, but was interrupted by the Japanese invasion and some records were lost. In the earlier paper both soil and botanical data were presented and it was concluded that, in determining the nutritional requirements of the rubber tree under Malayan conditions, the method of leaf analysis was a better indication of nutrient deficiency than soil analysis. In the work described here soil analysis was not used. With the exception of the peat area mentioned below, all the experiments were situated on granite and related soil types included amongst those described in the experiments on rubber. The soils of the area were surveyed by Cole (unpublished work), who also initiated the field experiments from which the samples were drawn.

In his review of the theory of foliar diagnosis, Thomas (1945) considered that the ratios of the nutrients to one another in the leaves were of the utmost importance. This was not found to be the case in rubber (Chapman, 1940) where the absolute amounts of nitrogen, phosphate, and potassium present determined the state of nutrition, but in the oil palm, as will be shown below, the state of nutrition of the plant in relation to potash and phosphate is determined largely by the ratio of K_2O to P_2O_5 present in the leaves; similarly the ratio of CaO to MgO is possibly of importance in determining the magnesium status, although data on this point are not conclusive.

Unlike the majority of plants investigated previously, the oil palm in Malaya shows little change with season, and produces new leaves at fairly regular intervals throughout the year, although there is a large seasonal variation in yield. The diurnal cycle of changes observed in some other plants (Penston, 1935; Phillis and Mason, 1942) is, however, also seen in the oil palm.

Owing to the method of growth of the palm, the factors leaf position and age, which were shown by the writer to be so important in rubber, cannot be separated. Leaf position alone is therefore referred to, the fronds being numbered from the youngest fully opened frond, but the age of the fronds is given approximately since middle-aged palms produce about 20 fronds per year. The great size of the fronds, which are usually over 15 ft. in length, introduces a variable usually neglected in small leaves, since whole fronds cannot conveniently be sampled and different parts of the frond vary greatly in composition. Even a single pinna is not of uniform composition.

A point of some importance is whether the constituents of leaf ash should be expressed on a basis of dry matter or ash. Thomas (1945) argues that the ash constituents should be expressed on dry weight because their concentration in the leaf determines growth. The latter statement is undoubtedly correct if by concentration effective concentration is meant, but it is not certain that the nutrient percentage in dry leaf matter is the better measure of effective nutrient concentration in the leaf. Phillis and Mason (1940) have concluded that base-exchange phenomena, similar to those demonstrated in soils, exist in the leaf. Until more is known concerning the nature of these adsorptions and to what extent some of the ash constituents may contribute to the adsorption of others, it cannot be concluded that either method of expression gives the better value for their effective concentration in the leaf. Presumptive evidence could be obtained on this point if either method of expression could be shown to give a better correlation with growth and yield. In the course of the present work a preliminary survey showed that the variation in ash percentage was seldom sufficient to give a significant difference between the two methods of expression. The use of leaf ash as a basis for expressing leaf-ash constituents does, however, save analytical work and was therefore adopted in this investigation.

Another interesting point is raised by the work of Hoagland and Arnon (1941), who showed that for mineral adsorption against a concentration gradient, energy provided by respiration is used. It is to be expected, therefore, that deficiency of a single nutrient or other unfavourable conditions which reduce growth and yield will often lead to a reduction in the supply of energy to the roots and therefore reduce their absorbing power. Thus a secondary correlation may be brought about between yield and nutrients which are not deficient in the rooting medium. In the work described below, the frequent correlation of nitrogen with yield is believed to be an example of this phenomenon, because no response was obtained to nitrogenous manuring but leaf nitrogen was increased by supplying whichever element was deficient.

SAMPLING AND ANALYTICAL METHODS

All samples were taken between 6.30 and 10.00 a.m. to avoid the effects of diurnal variation in leaf composition.

The analytical methods were the same as those used for rubber leaves in the earlier investigation except that, of the portion of the pinna selected for

analysis, only the midrib was rejected before analysis. Nitrogen (N) was estimated as such, phosphate as P_2O_5 , potassium, calcium, and magnesium respectively as the oxides.

NUTRIENT DISTRIBUTION IN THE FRONDS

Preliminary work on frond 1 indicated that there was a correlation between the yield from different plots and leaf composition. Young leaves are not in general convenient for routine sampling, however, because composition changes rapidly with development and considerable errors may result from sampling leaves of slightly different ages. In order to determine the most suitable material for sampling, a survey of the nutrient distribution in the palm leaves was necessary.

Variation in leaf composition may be considered under three headings:

1. Variation in composition between different parts of the same pinna.
2. Variation in composition of the same sections of pinnae in different parts of the same frond.
3. Variation in composition between different fronds, as determined by sampling a definite section of a definite pinna from each frond.

Since the oil palm has $3/8$ phyllotaxy, fronds 1, 9, 17, 25, &c., are beneath one another and can be readily located for routine sampling. Fronds from this series were therefore mainly used in this work. Each analysis was made on a composite sample from fifty palms.

The variation in different parts of the same pinna both longitudinally and transversely was determined for the central four pinnae of fronds 9 and 17. The longitudinal distribution was determined by dividing the pinnae into five equal transverse sections which were analysed separately after rejection of the midrib. The results are shown in Fig. 1, where the segments are numbered from the base to the tip of the pinna. Transverse variation is shown in Fig. 2, obtained from six longitudinal sections, three on either side of the midrib which was rejected. As might be expected, the pinnae from the older frond are lower in nutrients, but the changes in nutrient content with position on the pinnae are the same for fronds of both ages.

The concentration gradients for nitrogen (N) and potassium (K) in both figures are in opposite directions while phosphorus (P) occupies an intermediate position in Fig. 1, but follows potassium in Fig. 2.

It is significant that potash is lowest at the extremities of normal pinnae and that it diminishes with age, because it is at the extremities of the older pinnae that bronzing and finally necrosis starts during potash deficiency.

Fig. 3 shows the variation in the composition of the central 4 in. of the pinnae, with their position on the frond, for fronds 9 and 17. The fifteen points on each graph represent samples of equal size obtained by dividing all the pinnae of a frond into fifteen equal groups. Again it will be observed that the phosphate and potassium gradients are the reverse of the nitrogen gradient and that potassium is lowest at the ends of the fronds.

The above three figures enable a preliminary choice to be made of the best region to sample to determine nutrient deficiency and its relation to yield. All the curves in Fig. 1 are relatively flat in the region of section 3, while some indicate rapid changes in composition towards the bases and tips of the

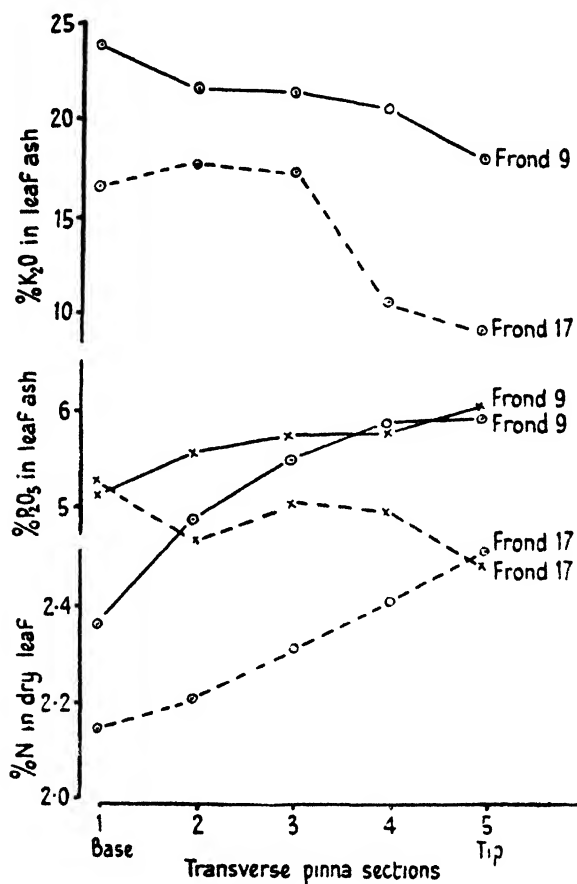


FIG. 1

pinnae. Similarly Fig. 3 shows that concentration gradients are minimal in the central regions of the fronds. In all work described below, therefore, the central 4 in. of the central two pairs of pinnae constituted the sample from a frond.

Fig. 4 shows the variation in the composition of fronds of different ages, from No. 1 to No. 37 for N, P₂O₅, K₂O, MgO, and K₂O/P₂O₅. The latter ratio is referred to below as *R*. The central 4-in. section of the central two pairs of pinnae was analysed in each case and the samples were taken from the two most divergent treatments of a field trial; one a control and the other heavily manured with phosphate and potash. The other three treatments gave intermediate curves. This manuring experiment gave no response to

either phosphate or potassium alone, but yield increase was only produced by the combination of rock phosphate and organic residues rich in potash. In this figure the phosphate and potassium curves are again similar and decrease at first rapidly and then more slowly with frond number, while

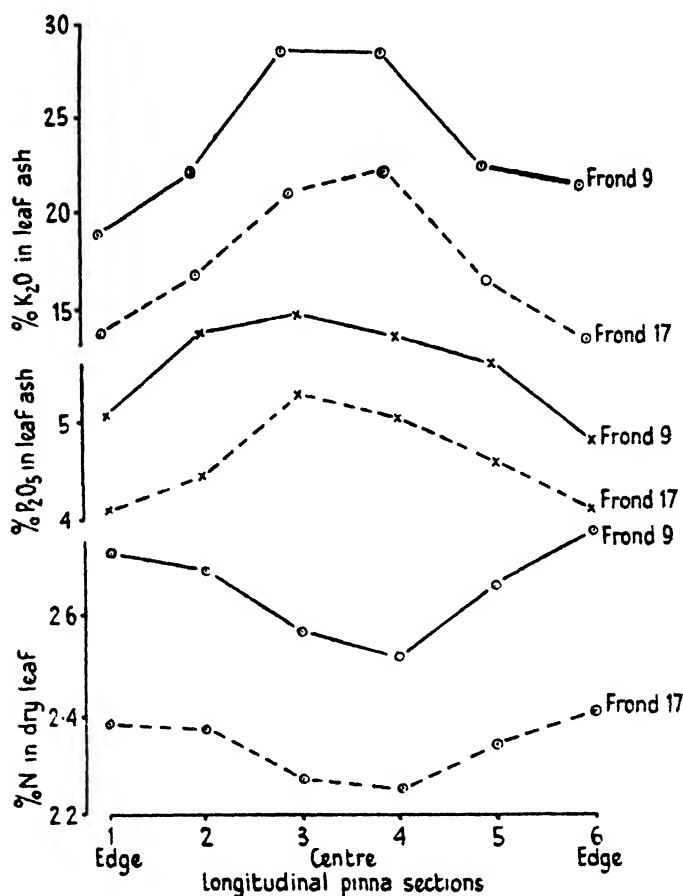


FIG. 2

nitrogen differs in first rising to a maximum. Further it will be observed that malnutrition has most effect on the composition of fronds of medium age which would therefore appear the most suitable for sampling. To confirm this, the correlation between yield and frond composition was investigated for fronds 1, 9, 17, and 25.

In Table I the correlation coefficients between yield and nutrient content for these fronds are shown. For nitrogen the correlation is good for fronds of all four ages, but for phosphate and potassium only the two oldest fronds show a significant correlation and between these last two fronds there is little to choose. Frond 17 was selected for further work because in some cases of acute deficiency frond 25 has necrotic areas.

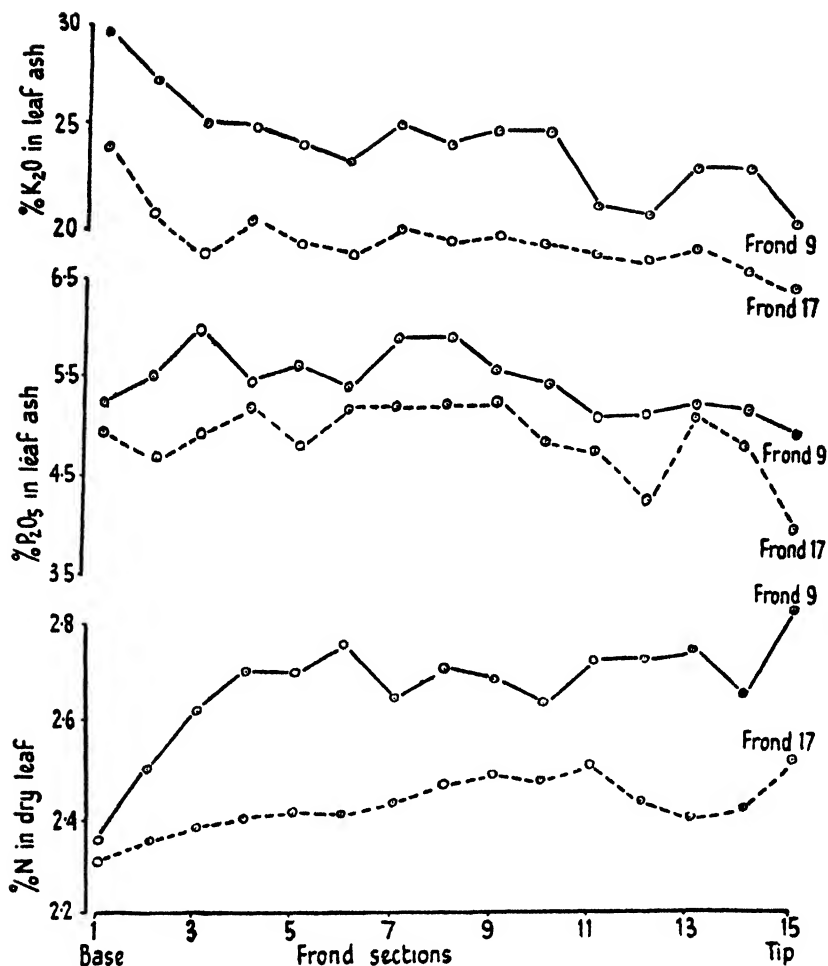


FIG. 3

TABLE I

Correlation Coefficient between Yield and Leaf Composition for Fronds of Different Ages

Frond no.	% N in dry leaf.	% P ₂ O ₅ in leaf ash.	% K ₂ O in leaf ash.
1	0.95	0.50	0.79
9	0.97	0.85	0.84
17	0.95	0.95	0.93
25	0.94	0.95	0.97

For $P = 0.05$ correlation coefficient = 0.88

In this and all succeeding tables values significant at $P = 0.05$ are in heavy type.

To determine the minimum number of palms which should comprise a single plot sample in an experiment, fronds 9 and 17 from 100 untreated

palms were analysed separately for phosphorus and potassium. The standard errors for 20 palm plots were as follows:

					% P_2O_5 in leaf ash.	% K_2O in leaf ash.
Fron	9	.	.	.	0.43	2.45
„	17	.	.	.	0.42	2.21

It will be observed that the standard errors for frond 17 are slightly lower than for frond 9. Twenty palm plots cover about 0.4 acre and were used as the minimum size whenever possible.

EFFECT OF NUTRITION ON LEAF COMPOSITION

As stated above, the ratio K_2O/P_2O_5 or R is of primary importance and the field experiments will be considered in order of increasing R values. The values of R for the control treatments are shown in Table II.

TABLE II
Leaf Composition of Field Experiments on Mature Palm

Expt. no.	Type of soil.	Average for control plots frond 17			Response in yield to.	Months required for response.
		% P_2O_5 in leaf ash.	% K_2O in leaf ash.	R .		
1	Peat	7.10	9.67	1.36	K (P depresses yield)	8
2	Medium loam	4.45	9.39	2.11	K	12
3	Medium loam	3.77	10.70	2.84	K and P	12
4	Clay loam	4.25	12.34	2.90	K and P	P 24 K 36
5	Sandy loam	3.85	14.52	3.76	P	9
6	Clay	3.46	13.93	4.03	P (K depresses yield)	9

In general with medium R values and low absolute percentages of phosphate and potassium, manuring with either of these substances alone produced yield increases, but with extreme R values depression in yield was produced by treatments which led to a displacement of R away from the optimum. Detailed records relating to experiments 2, 3, and 4 quoted in Table II are incomplete owing to enemy action and these experiments are omitted from the following discussion. In all cases significance is taken at the level of probability, $P = 0.05$, while for results referred to as strongly significant $P = 0.01$.

Experiment I

The experiment was situated on peat which had been cleared of jungle, drained, and planted about 8 years previously. Initially the palms grew fairly well, but when the experiment was started all except the youngest fronds were yellow and were dying prematurely from the tips.

The ash of several of these dying fronds contained less than 2 per cent. of potash, while phosphate remained abnormally high. A $2 \times 2 \times 2$ factorial experiment (presence and absence of N, P, K) with five replications was laid down in the area and the leaves were sampled a year later.

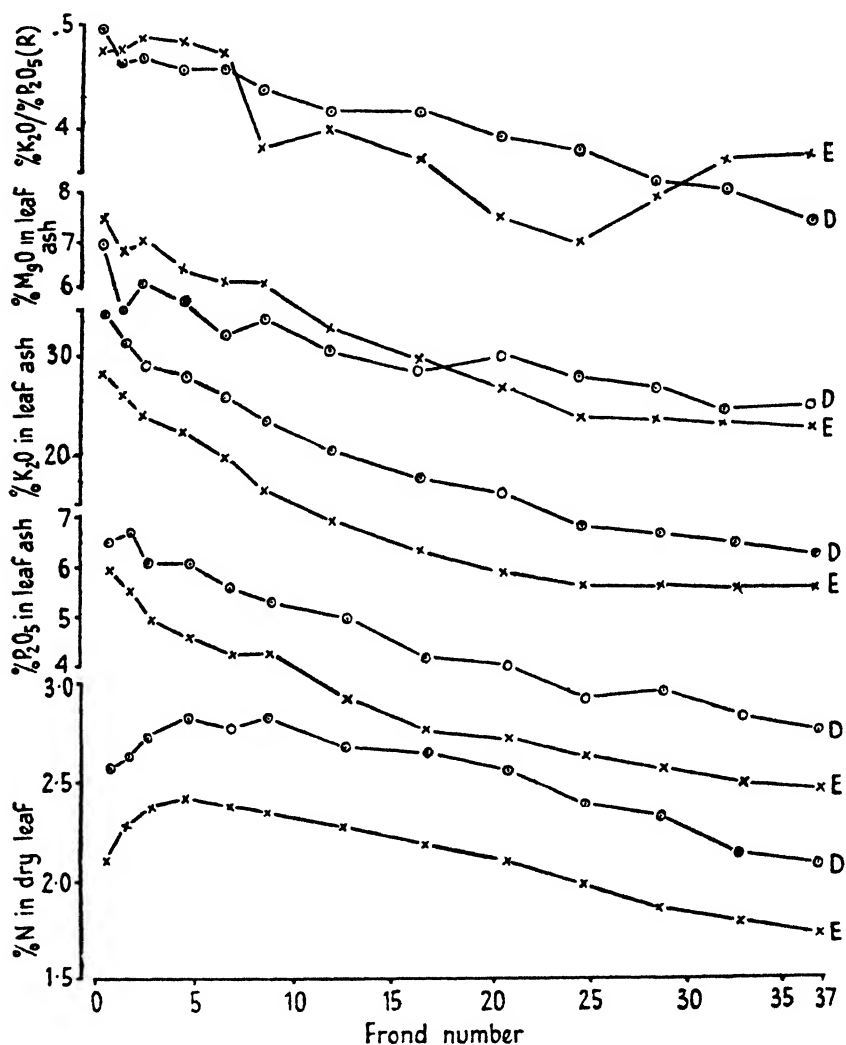


FIG. 4

Leaf composition 12 months after manuring is given in Table III together with yield for two periods each of 1 year, the first commencing 8 months and the second 14 months after manuring. The correlations between leaf constituents and yield during the second period are not significantly better than during the first, although the second period might be expected to give the higher correlation as the fruit takes 6 months from pollination to maturity, and the primordia are laid down more than 2 years before maturity.

From Table III it is seen that potash manuring has resulted in a large increase in leaf potash and yield, both the number and weight of the bunches having been increased. Calculated on treatment means, there are strongly significant correlations between leaf potash and yield (0.94) and between *R* and yield (0.94). The correlation between nitrogen and yield is 0.83 while phosphate and yield are not correlated.

TABLE III
Experiment 1: Leaf Composition and Yield

Treatment.	N	P.	K.	NP.	NK	PK	NPK.	C	Diff. for significance at 5% level.
% N in dry leaf	2.64	2.52	2.63	2.61	2.79	2.76	2.74	2.56	0.10
% P ₂ O ₅ in leaf ash	7.63	6.39	7.41	8.06	7.58	7.82	7.44	7.10	1.33
% K ₂ O in leaf ash	10.87	7.64	17.50	8.67	16.45	16.19	11.92	9.67	4.55
<i>R</i>	1.41	1.17	2.36	1.07	2.17	2.05	1.60	1.35	0.43
% CaO in leaf ash	19.44	16.05	15.74	17.18	14.56	15.85	15.77	18.36	1.62
% MgO in leaf ash	9.09	8.18	7.96	8.36	6.80	6.95	7.53	7.76	0.54
<i>1st period:</i>									
Yield % control	116.0	99.8	156.6	90.4	165.4	159.9	144.0	100.0	26.4
Bunch number % control	108.3	89.1	120.2	83.2	124.5	125.9	115.0	100.0	18.8
Average bunch weight % control	107.7	110.1	133.0	108.5	135.0	128.7	126.6	100.0	14.2
<i>2nd period:</i>									
Yield % control	112.2	91.6	170.4	84.4	179.9	163.6	135.8	100.0	28.4

Significance of Main Effects and NP Interaction

Treatment.	N.	P.	K.	NP	Diff. for significance at 5% level.
<i>1st period</i>					
Yield % control	-0.1	-11.5	54.8	-12.5	13.2
Bunch number % control	-1.1	-9.9	26.3	-7.2	9.4
Bunch weight % control	-1.5	-0.5	24.3	-3.5	7.1
<i>2nd period:</i>					
Yield % control	-3.3	-21.8	64.2	-14.2	14.2
<i>Leaf composition:</i>					
% N in dry leaf	0.08	—	0.15	-0.04	0.05
% P ₂ O ₅ in leaf ash	0.50	—	0.27	0.15	0.68
% K ₂ O in leaf ash	0.77	-2.52	6.30	-0.85	2.27
<i>R</i>	-0.17	-0.35	0.79	-0.11	0.21
% CaO in leaf ash	0.01	-0.59	-2.50	-0.29	0.81
% MgO in leaf ash	0.23	-0.14	-1.03	0.05	0.27

Correlation Coefficient between Yield and Leaf Composition

<i>1st Period:</i>			} Sig. (<i>P</i> 0.05) 0.71
Correlation between	% N in dry leaf and yield	0.57	
" "	% P ₂ O ₅ in leaf ash and yield	0.27	
" "	% K ₂ O in leaf ash and yield	0.98	
" "	<i>R</i> and yield	0.94	
<i>2nd period:</i>			
Correlation between	% N in dry leaf and yield	0.56	
" "	% P ₂ O ₅ in leaf ash and yield	0.28	
" "	% K ₂ O in leaf ash and yield	0.94	
" "	<i>R</i>	0.98	

Leaf nitrogen is correlated with yield although manuring with nitrogen did not increase yield. It should, however, be noted that potash manuring increased leaf nitrogen far more than did manuring with nitrogen. This is considered to be an example of the effect postulated above where increased

activity of the whole plant, brought about by supplying a deficient nutrient, leads to greater power of absorption.

Phosphate manuring produced a strongly significant depression in yield, and as in rubber (Chapman, 1941) a depression in leaf potash. A negative interaction between nitrogen and phosphate just attained significance while the field appearance of the plots receiving both nitrogen and phosphate was worse than the control.

This negative interaction between nitrogen and phosphate is probably due to increased phosphate absorption in the presence of nitrogen and to the accompanying decrease in potash absorption. Neither of these effects is significant by itself, but since they both tend to reduce yield the combined effect attains significance. This nitrogen-phosphate interaction has frequently been observed by the writer in the course of rubber leaf analysis, but, as in the present instance, the effect on leaf composition rarely attains statistical significance, although the effect on growth is often significant. But in the case of rubber it is positive because rubber in Malaya is generally deficient in phosphate.

These results differed from those obtained for rubber and for oil palms in other areas, in that phosphatic manuring did not increase leaf phosphate or potash manuring depress it. This and all other oil-palm experiments differed from those on rubber in that phosphate did not depress leaf nitrogen. An interesting feature of this reduction in yield due to phosphatic manuring was that it was entirely due to a reduction in the number of bunches of fruit, the weight of the individual bunches remaining constant. Since response to the manures commenced 8 months after application and it is over 2 years from the time the bunch primordia are laid down to the time of harvest, while sex determination takes place at an early stage, it is clear that the phosphate manuring induced abortion of the female bunches at a late stage in their development.

Calcium and magnesium figures are also given in Table II. Calcium is negatively correlated with yield, but the negative correlation of magnesium just fails to attain significance. This negative correlation is almost certainly of secondary origin due to the inverse correlation usually found between potassium and calcium. It will be observed that potash manuring has depressed both leaf calcium and leaf magnesium.

Experiment 5

In this experiment young mature palms planted on virgin jungle sandy loam were used. The treatments were the same as in expt. 1 and manuring was annual.

The results for yield during the third year, and leaf analysis at its commencement are shown in Table IV. Response to phosphate was noticeable 9 months after application, and by the third year yield on the phosphate plots had doubled, due to an increase in both the number and weight of the bunches. Potash and nitrogen as manures were without effect on yield, but while

leaf potash shows no correlation with yield, leaf nitrogen is again correlated with it.

TABLE IV
Experiment 5

Treatments.	N.	P.	K	NP	NK.	PK	NPK.	Control.	Sig diff. 5%.
% N in dry leaves	2.31	2.43	2.18	2.46	2.32	2.36	2.57	2.23	0.087
% P ₂ O ₅ in leaf ash	3.99	4.81	3.84	4.99	3.79	4.57	5.00	3.76	0.290
% K ₂ O in leaf ash	15.41	15.07	16.78	15.41	17.42	17.09	16.97	14.52	2.17
% Ash	7.21	7.32	7.36	7.20	8.10	7.15	7.26	7.84	0.93
R	3.87	3.13	4.37	3.10	4.60	3.74	3.39	3.87	0.55
% Control yield	124.2	209.1	94.8	190.9	103.6	186.4	207.8	100.00	30.6

Significance of Main Effects

% N in dry leaves	0.12	0.20	0.01						0.04
% P ₂ O ₅ in leaf ash	0.20	1.00	0.11						0.14
% K ₂ O in leaf ash	0.44	0.11	0.33						1.08
R	-0.04	-0.83	-0.16						0.29
Yield oil % control	9.2	92.9	7.9						15.3

Correlation between leaf N and yield	r =	0.886	
" " leaf ash P ₂ O ₅ and yield	" =	0.973	
" " " K ₂ O " "	" =	-0.011	
" " R and yield	" =	-0.863	0.71
" " P ₂ O ₅ in dry leaf and yield	" =	0.930	
" " K ₂ O " "	" =	-0.350	

The percentage of leaf ash has been little affected by the treatments and hence the correlation coefficients for yield with phosphate and potash are not altered significantly, whether they are based on leaf ash or dry matter values, for these two nutrients.

Experiment 6

The treatments in this experiment were the same as those in expts. 1 and 5, but it was situated on a medium clay, the heaviest soil in this series of experiments. The first leaf sample was taken before the second annual application of manure. The results are shown in Table V. Yield has

TABLE V
Experiment 6

Treatment.	N	P.	K.	NP.	NK	PK	NPK	Mg B	Control	Sig diff. 5%.
% N in dry leaves	2.40	2.47	2.40	2.52	2.47	2.46	2.40	2.53	2.46	0.07
% P ₂ O ₅ in ash	3.75	4.01	3.73	4.22	3.76	3.96	4.05	4.22	3.91	0.25
% K ₂ O in ash	16.05	15.28	16.39	14.74	16.88	15.12	15.74	15.77	15.69	1.65
R	4.28	3.81	4.39	3.50	4.40	3.82	3.80	3.74	4.01	0.41
% Control yield	94.1	124.0	87.6	117.2	94.2	107.4	110.6	111.8	100.0	15.0

Significance of Main Effects

% N in dry leaves	0.043	0.033	-0.029							0.039
% P ₂ O ₅ in ash	0.041	0.268	-0.004							0.131
% K ₂ O in ash	0.229	-1.032	0.503							0.866
R	0.019	-0.525	0.245							0.217
Yield oil % control	-0.7	20.9	-8.9							7.8

Correlation between Leaf N and yield	=	0.58	
" " leaf ash P ₂ O ₅ and yield	" =	0.83	
" " " K ₂ O " "	" =	-0.81	0.67
" " R and yield	" =	-0.88	

not been significantly altered by nitrogenous manuring, but phosphate has increased yield and potash has depressed it. Phosphate has decreased leaf potash and increased leaf phosphate so that R has been considerably reduced. The depression of yield by potash is seen to be due to an increased value of R , i.e. the ratio K_2O/P_2O_5 . This increase in R by potash has been produced by two additive effects neither of which attains statistical significance alone: a depression in leaf phosphate and an increase in leaf potash. Leaf phosphate is seen to be positively correlated with yield, while leaf potash and R are negatively correlated.

Following the second annual application of manure, leaf samples were taken periodically. The original leaf sample taken just before the second application of manure showed the effect of the first application and in subsequent samplings the effect of the second application was superimposed on that of the first. The effect of the second application is given by the difference in leaf composition at the first and subsequent samplings. The main effects are given in Table VI. The interactions are not given as none attained significance.

TABLE VI
Experiment 6: Effect of Manuring with

Weeks after manurial application.	Treatment.			Significance 5% level.
	N.	P.	K.	
<i>Leaf N</i>				
4 weeks	0.015	0.009	—0.031	0.047
8 ,,	0.010	0.055	0.029	0.053
19 ,,	0.021	0.058	0.019	0.037
30 ,,	0.005	0.081	—0.004	0.039
<i>Leaf Ash P₂O₅</i>				
4 weeks	0.042	0.071	—0.064	0.132
8 ,,	0.082	0.333	—0.058	0.193
19 ,,	—0.020	0.409	—0.029	0.179
30 ,,	—0.043	0.246	0.009	0.132
<i>Leaf Ash K₂O</i>				
4 weeks	—0.455	0.136	—0.440	0.566
8 ,,	—0.710	0.446	—0.569	0.745
19 ,,	0.665	—0.025	—0.152	0.719
30 ,,	—0.560	—0.218	0.188	0.560

It will be observed that neither N nor K have had any effect on leaf composition during 7 months, although they were both applied as the readily soluble sulphates. But phosphate which was applied as the relatively insoluble rock phosphate became effective in 8 weeks, increasing both leaf phosphate and leaf nitrogen. It would therefore appear that the requirement of the plant rather than the solubility of the fertilizer determined its leaf concentration in this experiment. This result should be compared with those obtained in expt. 1. These are the only two experiments in which manuring with an element in excess of requirement did not increase its concentration

in the leaf and in both experiments the excess reduced yield. In expts. 1, 5, and 6 leaf nitrogen was increased by whichever of the two nutrients, phosphate or potash, was deficient and has been for this reason correlated with yield.

Experiment 7

This randomized block replanting experiment contained 100 10-palm plots and 10 treatments. Except in treatment 9 the manures were applied per plot every 6 months as follows:

1. 100 lb. fruit bunch waste.
2. 100 lb. fruit bunch waste + 1 oz. N + 1 oz. P.
3. 1.67 lb. bunch ash equivalent to 100 lb. fruit bunch waste + 1 oz. N + 1 oz. P.
4. 1 oz. P.
5. 1 oz. N + 1 oz. P.
6. 1 oz. N + 1 oz. P + 1 oz. K.
7. 50 lb. composted fruit bunch waste equivalent to 100 lb. bunch waste.
8. 1 oz. N + 1 oz. P + 1 oz. K + 2 oz. subsidiary mineral mixture containing the following proportions of the hydrated crystals:

Magnesium sulphate	90 parts.
Ferrous sulphate	4 "
Borax	4 "
Copper sulphate	1 part
Manganese sulphate	1 "

9. 1 oz. N + 1 oz. P + 1 oz. K at 3-monthly intervals.
10. Control.

N was supplied as ammonium sulphate, P as Gafsa rock phosphate, $8\frac{1}{2}$ oz. = 1 oz. P, and K as potassium sulphate.

Before planting, the young plants were grown for a year in a nursery in good soil, so that the full effect of the treatments was not apparent during the first year after planting. Measurements of growth and leaf samples were taken during the first 3 years. The results are shown in Table VII, where analyses for frond 1 instead of frond 17 are given for the first year because of the difficulty of determining accurately the position of the older fronds in very

TABLE VII

Experiment 7: Leaf Composition of Young Palms

Treatment	1.	2	3	4	5	6	7.	8	9.	10
No. of fronds % control	106.8	112.7	111.8	101.4	108.2	105.9	108.2	104.5	110.5	100.0
No. of leaflets % control	103.3	113.3	113.0	105.8	104.2	106.8	112.9	108.1	109.0	100.0
Frond length % control	116.2	120.3	117.5	105.3	107.0	111.4	118.1	111.6	122.4	100.0
% N in dry leaf, frond 1 (Nov. 1939)	2.58	2.60	2.61	2.53	2.82	2.05	2.54	2.70	2.68	2.52
% P_2O_5 in leaf ash, frond 1 (Nov. 1939)	7.47	8.41	7.51	9.18	11.01	8.20	7.63	8.16	8.02	8.64
% K_2O in leaf ash, frond 1 (Nov. 1939)	37.48	37.92	35.74	29.85	26.92	34.85	34.59	34.72	37.38	30.27
R	5.02	4.51	4.76	3.25	2.45	4.25	4.53	4.25	4.66	3.50
Height % control	136.6	145.7	140.7	111.1	120.7	130.0	135.2	130.5	141.9	100.0
Spread % control	128.4	138.1	136.3	106.7	119.3	122.6	132.2	126.3	135.7	100.0
% N in dry leaf, frond 17 (Nov. 1940)	1.95	2.20	2.14	1.84	2.04	2.02	1.91	2.20	2.14	1.82
% P_2O_5 in leaf ash frond 17 (Nov. 1940)	4.56	5.35	4.66	4.59	5.03	4.80	4.47	5.46	5.30	4.17
% K_2O in leaf ash, frond 17 (Nov. 1940)	32.28	31.30	27.17	12.70	10.67	20.16	22.40	20.83	24.61	12.71
R	7.08	5.85	5.83	2.77	2.12	4.20	5.03	3.82	4.64	3.05

TABLE VII (cont.)
Correlation Coefficient for Treatment Means Between

	Sig. ($P < 0.05$)	0.632
Frond numbers and % N in dry leaf, frond 1 (Nov. 1939)	0.338	
„ % P_2O_5 in leaf ash, frond 1 (Nov. 1939)	0.559	
„ % K_2O in leaf ash, „ „	0.569	
Frond length and % N in dry leaf, „ „	-0.523	
„ % P_2O_5 in leaf ash, „ „	-0.688	
„ % K_2O in leaf ash, „ „	0.609	
Number of pinnae and % N in dry leaf, frond 1 „	-0.084	
„ % P_2O_5 in leaf ash, „ „	-0.552	
„ % K_2O in leaf ash, „ „	0.711	
Height of palms and % N in dry leaf, frond 17 (Nov. 1940)	0.743	
„ % P_2O_5 in leaf ash, „ „	0.555	
„ % K_2O in leaf ash, „ „	0.927	
„ R	0.753	
Spread of palms and % N in dry leaves, frond 17 (Nov. 1940)	0.764	
„ % P_2O_5 in leaf ash, „ „	0.553	
„ % K_2O in leaf ash, „ „	0.810	
„ R	0.714	
Height of palms and % P_2O_5 in leaf ash, frond 17 (Nov. 1941)	0.391	
„ % K_2O in leaf ash, „ „	0.762	
„ R	0.709	

young palms. It is clear from the previous discussion of Fig. 4 that direct comparison cannot be made between these values and those obtained from frond 17.

By the end of the first year some correlation was shown between growth and leaf potash, the number of pinnae of frond 1 was correlated with leaf potash, while the length of frond 1 (frond length) and the total number of fronds (frond number) just failed to attain a significant correlation. Phosphate was correlated negatively with frond length.

As a measure of growth rate at the time of sampling, number of pinnae \times area of pinnae for the first mature frond was used. The following correlations with leaf composition were obtained:

	Correlation coefficient.
% N and growth	-0.20
% P_2O_5 and growth	-0.73
% K_2O and growth	0.79

The negative correlation with phosphate is particularly interesting, as a response to phosphate was shown later for those treatments which received heavy dressings of K. The R values shown for frond 1 were considerably higher than they would have been had frond 17 been obtainable, as can be seen by comparing the R values for different fronds in Fig. 4. In the second series of samples (Oct. 1940), however, some of the R values were even higher, although frond 17 was used, owing to the very heavy potash manuring in all treatments receiving fruit bunch waste and treatment 9 with 3-monthly applications of $N+P+K$; and in these treatments phosphate had now become the limiting factor, as can be seen from the following figures for the best five treatments, arranged according to their increased height as compared with control.

Treatment no.	R.	Leaf ash % P_2O_5 .	Leaf ash % K_2O .	Height % control.
2	5.85	5.35	31.30	145.7
9	4.64	5.30	24.61	141.9
3	5.83	4.66	27.17	140.7
1	7.08	4.56	32.28	136.6
7	5.03	4.47	22.49	135.2
Correlation with height	-0.161	0.895	0.335	

The correlation of height with leaf ash P_2O_5 is now positive and significant, while R and K_2O do not give significant correlations.

From the second year onwards yellowing of the older fronds appeared, especially in treatments 5, 6, and 9, and at the end of the third year frond 17 was sampled; the results of the analysis are given in Table VIII, from which

TABLE VIII

Experiment 7

Treatment	% CaO in leaf ash.	% MgO in leaf ash.	CaO/MgO.	% Chlorotic palms.
1 . . .	13.96	5.24	2.66	0
" 2 . . .	16.08	5.12	3.14	0
" 3 . . .	15.92	4.66	3.42	2
" 4 . . .	20.28	3.63	5.59	25
" 5 . . .	21.56	3.48	6.20	50
" 6 . . .	20.92	3.09	6.77	44
" 7 . . .	17.47	6.21	2.81	0
" 8 . . .	19.92	4.43	4.50	4
" 9 . . .	21.06	2.41	8.74	70
" 10 . . .	18.63	3.77	4.94	17

it is clear that low leaf magnesium characterizes the chlorosis. It will further be observed that all treatments which did not receive magnesium either with the chemical fertilizers or as fruit bunch waste developed some chlorotic plants. The worst treatments were those in which the plants received ammonium or potassium sulphates without any form of magnesium (see Fig. 5). This effect of sulphates is well known in 'sand drown' of tobacco (Garner, McMurtrey, Bacon, and Moss, 1923). Post-war work by the writer on various other species has shown that a chlorosis of the older leaves characterized by a low leaf magnesium and a high CaO/MgO ratio can be cured by treating the soil with magnesium compounds. In Fig. 5 calcium, magnesium, and the ratio CaO/MgO are shown against the percentage of plants affected in each treatment. The treatments which received ammonium sulphate are marked S. It would appear that the relationship between the incidence of chlorosis and magnesium content of the leaf ash is not truly linear, but it is sufficiently nearly so to give the significance value of -0.86 for the correlation coefficient between disease incidence and leaf magnesium. Similarly the correlation coefficient between CaO/MgO and disease incidence was also high, viz. 0.91.

It is clear from the figure that all the points, including those marked *S*, belong to the same curve. From this it must be concluded that ammonium sulphate intensifies chlorosis solely by lowering leaf magnesium.

It will be recalled that in Fig. 4 MgO was shown to fall with leaf age: this fact is undoubtedly related to the incidence of magnesium deficiency chlorosis

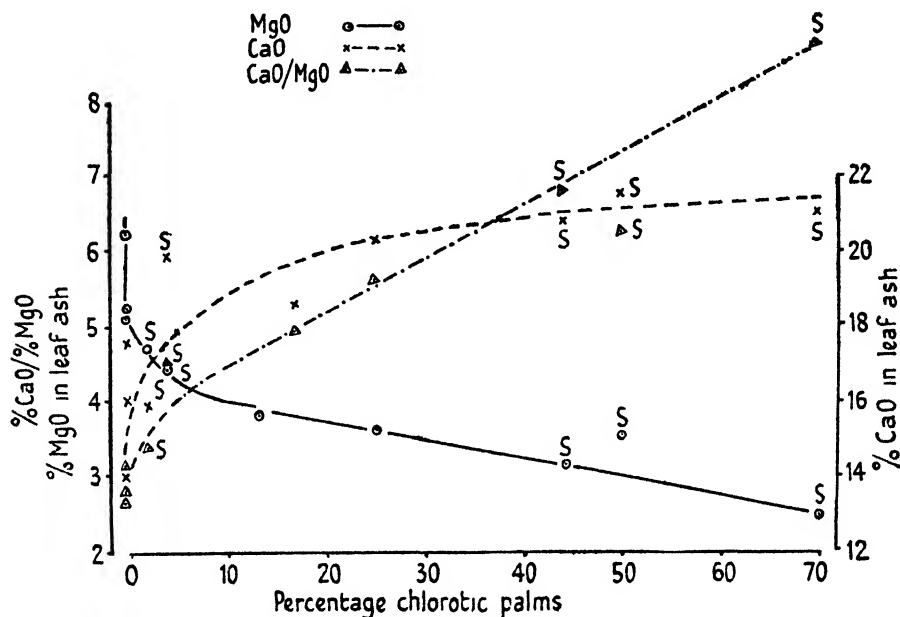


FIG. 5

in the older leaves, and may be compared with the distribution of potash in palm leaves and the appearance of potash deficiency symptoms described above.

DISCUSSION

Several interesting points on the theory of the relation of leaf composition to plant nutrition are raised by the work described above. Although leaf nitrogen is often correlated with yield, no increase in yield was obtained from nitrogenous manuring. Indeed, leaf nitrogen was in one experiment increased as much by manuring with potash as by manuring with ammonium sulphate. Extreme caution must therefore be used in deciding whether in any given example a low value for a nutrient is the cause or the effect of malnutrition. It is now recognized (Goodall and Gregory, 1947) that the expenditure of energy obtained from respiration is often required for absorption by the roots. In such cases it must be expected that factors affecting the general health of the plant such as carbon nutrition and light intensity, on which the energy-supply to the roots depends, will also influence the amount and ratio of the elements entering the plant and finally found in the leaves.

In this series of experiments, although nitrogen deficiency in the soil was not encountered, there was a wide range from deficiency to excess for both phosphate and potassium. It is at both extremes of this range that the value of R becomes important for growth and yield.

From Table II it is clear that the optimum value of R for mature palms lies approximately in the range between 2.5 and 3.5. With R below 2.5, potassium has proved the deficient nutrient, the leaf potash being correlated with yield, while with even lower R values (e.g. 1.36) the deficiency of potassium is so great that application of phosphate has actually depressed yield. With R values above 3.5 phosphate is the deficient nutrient, the leaf phosphate being correlated with yield. With even higher R values (e.g. 4.03) the deficiency of phosphate is so great that application of potash has depressed yield. Examination of Table IV shows the two highest yielding plots with R values of 3.13, 3.39 respectively, which may be taken as the upper limit of the optimum. There is some indication that the optimum value of R may vary slightly with the absolute quantities of phosphate and potassium present. From expt. 7 the value of R for the growth of immature palms would appear to be higher. The best five treatments have R values ranging from 4.64 to 7.08; only above this lower value for R is there a response to phosphate, while there is no response to potash. This lower value therefore gives the upper limit of the optimum value for R . No data on the value for the growth of mature palms are available.

The incidence of magnesium deficiency is equally highly correlated with the absolute amount of magnesium in the leaves and with the CaO/MgO ratio, so that it cannot be stated whether this ratio is of importance to the nutrition of oil palms. In this work all palms in each plot were sampled and the composition of the bulked samples was compared with the percentage of chlorotic palms in the plot. In cases of this kind, where there is an apparent sharp distinction between normal and diseased plants, comparison is sometimes made between the compositions of the two groups. This has recently been done for oil palms by Hale (1947), and the hope has been expressed by this writer that it may be possible to find an 'ideal' composition for normal palms, although the normal palms from two diseased areas compared differed considerably in composition. As long ago as 1931 Chapman drew attention to the fact that normal plants growing in an area where iron-deficiency chlorosis was severe approximated in composition to chlorotic plants in an area where the incidence of the disease was slight. It cannot be assumed that the probability of any given plant developing deficiency symptoms is the same as that for any other and bears no relation to its individual constitution; thus the normal plants in an area of malnutrition will not be a random sample of the population. Therefore the lower the incidence of normal plants in the general population of an area the more will their average composition differ from that of normal plants in an area where all plants are healthy. With extremely homogeneous horticultural varieties it may be possible to neglect individual variation, but it has been shown above that the standard error of single-palm plots is high.

From the above considerations it is clear that normal oil palms from two areas where the incidence of malnutrition is very different cannot be expected to have the same leaf composition. Where it is desired to compare normal with deficient palms, both must occur in the same area and due weight must be given to the limitations implied by statistical considerations.

SUMMARY

1. A survey of the nutrient content of the fronds of normal palms was carried out for nitrogen, phosphate, and potassium. It was found that all nutrients varied in concentration not only in different parts of the same frond but in different parts of the same pinna. Nitrogen tended to rise towards the extremities of both fronds and pinnae, while potash showed a very sharp fall at all extremities.

Phosphate occupies an intermediate position. Except for nitrogen which shows a slight initial rise, nitrogen, phosphate, and potash all decrease in fronds older than the first fully opened frond.

2. Attention is drawn to the fact that, in potash deficiency, discoloration and finally necrosis appear first in the regions which are lowest in potash. Chlorosis due to magnesium deficiency similarly appears first in the older leaves which are lower than the younger in magnesium. Senescence similarly appears first where nitrogen, phosphate, and potassium are lowest.

3. The correlation of yield with the composition of samples taken from the central pinnae of various fronds was compared, and on the basis of this comparison frond 17 was selected for routine sampling.

4. The standard error for determinations of phosphate and potash was determined using 100 single-palm plots, and twenty-palm plots were selected for routine work.

5. It is shown that the ratio K_2O/P_2O_5 (R) is of primary importance in determining the response to potash or phosphate manuring, and that the optimum value of R for yield lies in the range between 2.5 and 3.5.

6. With low values for R (below 2.5) potassium was the deficient nutrient, and its use as manure increased both yield and leaf potash, which therefore showed a significant correlation. Manuring with phosphate had no effect on yield except where the R value was extremely low, when phosphate depressed yield and also reduced leaf potash, without increasing leaf phosphate significantly. Manuring with potassium salts in addition to increasing leaf potash also increased leaf nitrogen as much as did manuring with nitrogen.

7. With high values for R (above 3.5), phosphate was the deficient nutrient, and its use as manure increased both yield and leaf phosphate, which therefore showed a significant correlation. Manuring with potassium had no effect on yield except where the R value was extremely high, when yield was depressed. This depression in yield produced by potassium manuring is attributed to two additive effects, neither of which attained significance alone, a depression in leaf phosphate and an increase in leaf potash which resulted in a significant increase in R . With very high R values (extreme deficiency of phosphate)

neither nitrogen nor potash was increased in the leaves within 7 months by applications of sulphate of ammonia or sulphate of potash, although the relatively insoluble rock phosphate increased leaf phosphate in 8 weeks after application.

8. Although leaf nitrogen was so often correlated with yield, manuring with nitrogen never increased yield.

9. In immature palms growth is shown to be correlated with leaf composition. With high *R* values growth is correlated with phosphate content and with low values with potash content.

10. The incidence of magnesium deficiency was shown to be related both to absolute leaf magnesium and to the ratio CaO/MgO . Ammonium sulphate intensifies magnesium deficiency by reducing leaf magnesium.

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On the Genus *Resticularia*

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With four Figures in the Text

INTRODUCTION

THE genus *Resticularia* with the single species *R. nodosa*, a parasite within the trichomes of the blue-green alga *Lyngbya aestuarii*, was erected by P. A. Dangeard in 1890. He suggested that the genus should be placed not far from *Lagenidium* and *Myzocytium*. The thallus of *R. nodosa* formed an unbranched tube piercing each cell throughout the whole length of the parasitized filament. From the parasite exit tubes were developed, as in *Lagenidium*, through each of which an undifferentiated spherical protoplasmic mass was discharged which later cleaved into a small number of zoospores. In connexion with these Dangeard remarked 'autant que nous avons pu en juger, elles n'ont qu'un long cil, traîné à l'arrière', but no figures of the zoospores were given. Resting spores were produced in an intercalary position at intervals along the thallus. These had thick, smooth, colourless walls, granular contents with one large eccentric oil drop in each spore. He regarded the resting spores as zygospores and, if his account of their formation were correct, it would be a unique process. It appeared that the contents of the much-elongated thallus aggregated at certain points so that at intervals there were paired masses of protoplasm. In each pair the two masses (gametes) were of much the same size, and the sexual process appeared to be isogamous, the two gametes uniting to give a zygospore. Neither in connexion with this sexual process nor in connexion with zoospore formation did Dangeard observe any cross-walls in the thallus.

In 1903 Fritsch found a fungus parasitic within the trichomes of *Tolypothrix* sp. producing intercalary resting spores. He, not unnaturally, identified this as *R. nodosa*. However, Fritsch's fungus showed very striking differences from Dangeard's organism. The thallus was divided into a large number of short cells at maturity, the resting spore was dark brown without a single dominant oil globule, and there was no suggestion that it was formed as the result of a sexual process. It was simply a chlamydospore. But, most outstanding of all, no trace of zoosporangia or of zoospores was to be seen. Fritsch's fungus showed a pronounced tendency to grow out of the trichomes into the surrounding water as a fine branched mycelium on which the dark-brown chlamydospores were developed on short lateral branches. The extramatrical mycelium apparently provided the means by which the fungus spread from diseased to healthy filaments of *Tolypothrix*.

On the basis of his observations Fritsch published an emended diagnosis of *R. nodosa* which more or less combined his observations with those of Dangeard. He further described a second species, *R. Boodlei*, also in *Tolythrix* sp. This had an endophytic mycelium without thick-walled resting spores, an ectotrophic mycelium which was relatively wide and, above all, this mycelium produced numerous large thin-walled conidia mostly in chains on lateral branches.

It is clear that *R. Boodlei* shows no feature which suggests that it belongs to the Phycomycetes, and the same is true of the fungus which Fritsch identified as *R. nodosa*. If Dangeard's account of *R. nodosa* had not been before him, Fritsch would, doubtless, have referred both fungi to the Fungi Imperfecti.

The drastic differences between the original account of *R. nodosa* and the account given by Fritsch have led those who have monographed the Phycomycetes to adopt an attitude of reserve towards the genus *Reticularia* and to regard it as a genus of very doubtful systematic position.

In the past 40 years very little has been added to our knowledge of the genus. Skvortzow (1925) described a new species, *R. Oedogonii*, on *Oedogonium*. His single indefinite figure and his inadequate diagnosis make it difficult to picture his fungus. It apparently formed resting spores singly in the *Oedogonium* cells and connected by a mycelium running longitudinally in the host filament. It is clear that insufficient is known about this fungus to classify it, and to place it in the genus *Reticularia* merely leads to confusion.

Sparrow (1932) found and figured an unbranched mycelium in *Tolythrix* sp. and referred it tentatively to *R. nodosa*, but no identification was really possible as it did not produce spores of any kind.

Couch (1941), in a paper on the action of the flagella in the zoospores of Phycomycetes, refers to observations on the biflagellate zoospores of *Reticularia* sp., figuring a zoospore with two flagella, one of the whip-lash type and the other of the tinsel type. This kind of zoospore is characteristic of Lagenidiales, Saprolegniales, and Peronosporales.

Karling (1942) in his monograph of biflagellate holocarpic Phycomycetes says in discussing *Reticularia* and its position in the Lagenidiaceae: 'Observations on a parasite found in Lyngbya in the laboratories at Columbia suggest that the organism found by Dangeard is a valid member of the family.' He gives no details of these observations and continues to list *Reticularia* as a doubtful genus of the family. He further gives as his opinion that 'the fungus which Fritsch described as *R. nodosa* as well as *R. Boodlei* in species of *Tolythrix* do not relate to *Reticularia* in the sense of Dangeard'. With this opinion I am in agreement.

OBSERVATIONS ON *RETICULARIA NODOSA*

In April 1948 I observed *Lyngbya* sp., collected from a pond in Sevenoaks, Kent, heavily parasitized by an endophytic fungus which agrees so closely

with Dangeard's description of *R. nodosa* that I have little doubt it is indeed that species.

Trichomes of the alga are nearly always attacked at their ends where the zoospore comes to rest and puts out a tube (Fig. 1) which penetrates the terminal cell and rapidly extends throughout the length of the trichome. The parasitized cells are killed and change from an indeterminate dirty greenish-grey to a pale yellowish-green. Further, staining with iodine indicates that the abundant supply of glycogen in the healthy alga is used up in the cells traversed by the fungus. The contents of the fungus are clear and rather shining with occasional scattered globules, probably of oil. Within the host trichomes the outline of the fungus is very irregular. In its early stages it tends to be somewhat moniliform, being constricted at each passage through a host cell-wall. Later, numerous small protuberances or diverticula develop which can hardly be called branches. Indeed, the mycelium appears to be typically unbranched. Usually a single trichome is occupied by an individual parasite, but at times two or more parallel thalli may be seen in the same trichome. Where, within the same sheath, two hormogones of the alga occur separated by an interval of empty sheath, the parasite grows as a rather narrow hypha with parallel sides (Fig. 1) very unlike the form assumed when host cells are traversed. There does not appear to be any tendency for the fungus to grow out from a trichome as an extra-matrical mycelium. Passage of the fungus from one individual of *Lyngbya* to another seems to depend solely upon zoospores.

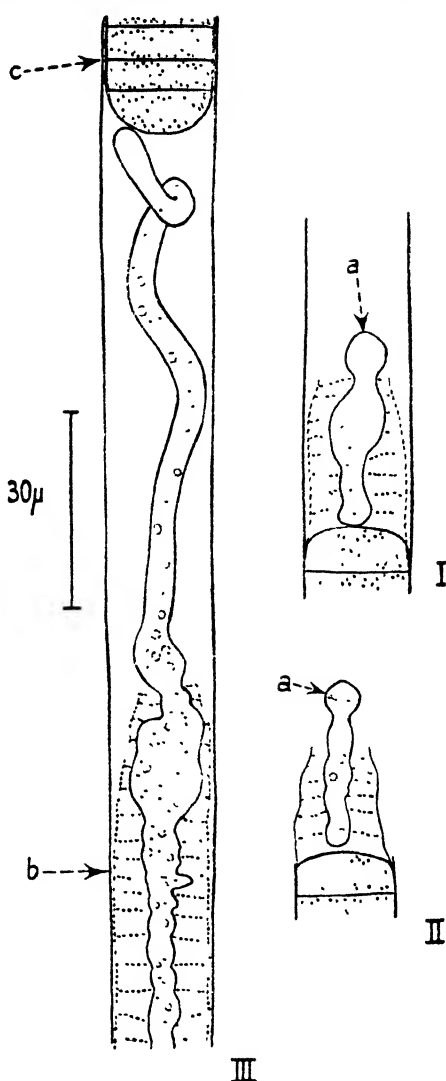


FIG. 1. *Lyngbya* sp., shown in optical section, attacked by *Resticularia nodosa*. I and II, Early stages in infection showing the germinating zoospore (*a*). The host cells penetrated by the germ-tube are dead and shrunken, but the host cells just in front of this tube are still alive and turgid. III, Young thallus of the fungus showing irregular form where host cells are traversed, and the even width developed in the space between two hormogones; (*b*) dead host cells, (*c*) living host cells.

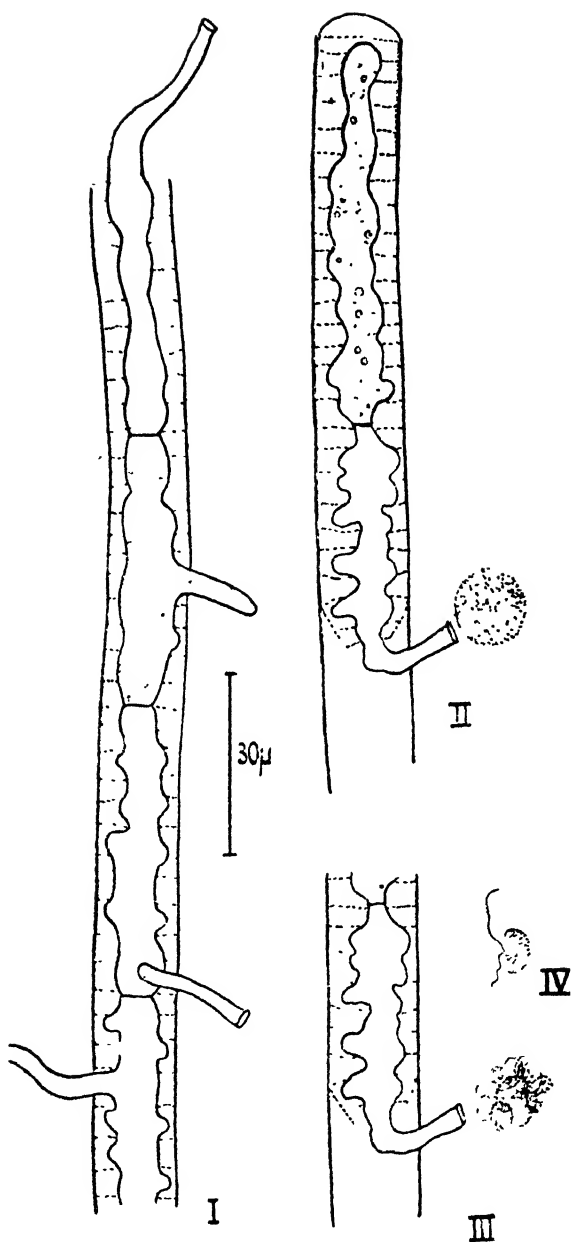


FIG. 2. *Lyngbya* sp., attacked by *Resticularia nodosa*, showing zoosporangia. I, Three empty zoosporangia and one young one. II, A zoosporangium is shown from which the protoplasmic contents have escaped, the limiting vesicle around the protoplasmic mass not being visible. III, The same, 5 minutes later after the protoplasmic mass had cleaved into seven zoospores. IV, A single zoospore.

When mature the thallus is partitioned by occasional cross-walls and each cell thus formed would seem to be destined to become either a zoosporangium or a sexual organ. The fungus would thus appear to be holocarpic.

Roughly two forms of zoosporangium can be recognized, but transitions occur and there is certainly no fundamental difference between them. In the first the sporangium is a simple segment of the thallus which develops an

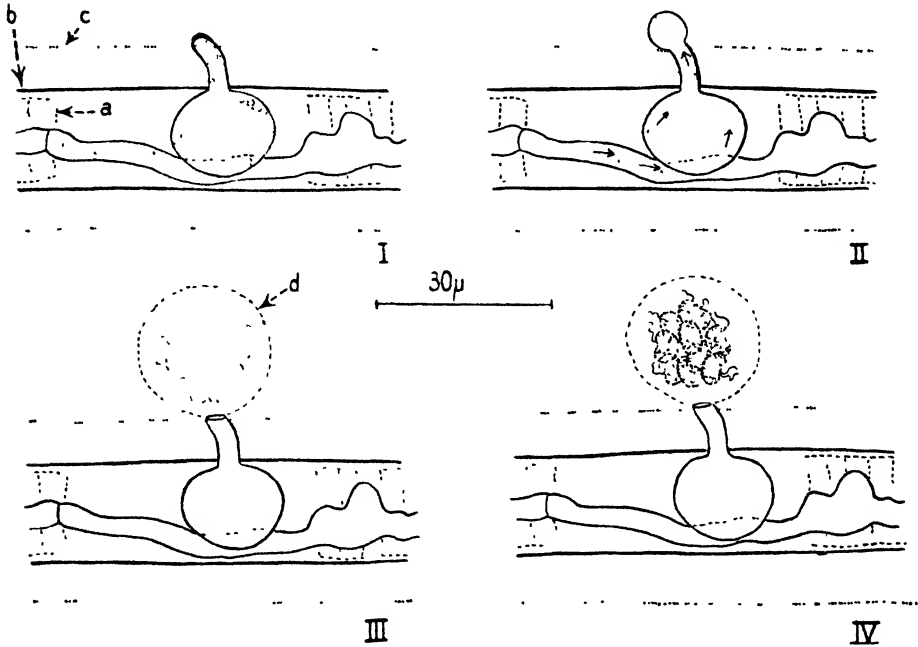


FIG. 3. Part of a diseased trichome of *Lyngbya* sp., seen in optical section, containing *Resticularia nodosa*. A zoosporangium is shown at various stages (I, II, III, IV) of zoospore formation. The sporangium consists of a length of thallus together with a spherical diverticulum furnished with an exit tube. One of the two limiting transverse walls of the sporangium is seen on the left. The sporangium occurs in an empty part of the trichome between two hormogones: (a) host cells; (b) firm sheath of host trichome; (c) limit of outer mucilaginous sheath, (d) the very thin wall of the vesicle surrounding the zoospore mass and here indicated diagrammatically by an interrupted line. The arrows in II show the direction of flow of the protoplasm during vesicle formation. Approximately 20 minutes elapsed between stages I and IV.

exit tube (Fig. 2). In the second the main body of the sporangium is formed from a diverticulum which becomes much inflated to form a spherical body from which the exit tube develops (Fig. 3).

The exit tube projects a short distance beyond the firm sheath of the host filament and sometimes it can be seen that the degree of projection corresponds with the thickness of an additional sheath of slime which is, however, difficult to see under the microscope.

Fig. 3 shows stages in vesicle formation and zoospore delimitation. The tip of the exit tube is somewhat thickened. Quite suddenly, no doubt as a result of the softening of this tip, a vesicle is blown at the end of the exit tube,

the very thin wall being formed of the material of the softened tip. The finely granular protoplasm may be seen rushing out of the sporangium (as indicated by the arrows in Fig. 3) into the vesicle, which reaches its full size within the space of a few seconds. When the vesicle is fully formed the original sporangium is empty of granular material and the vesicle is filled with a finely granular mass of undifferentiated protoplasm which very soon retracts from the vesicle wall, ceases to be spherical, and shows movements of a somewhat amoeboid nature. During the next 10 minutes the protoplasmic mass cleaves into 7–10 bean-shaped zoospores each with two lateral flagella. The zoospores show considerable activity within the vesicle. Then, after a further 10–15 minutes, its wall breaks down and the zoospores swim rapidly away. The vesicle wall is extremely thin and is sometimes impossible to see under the microscope, but even then its existence can be inferred firstly because the extruded protoplasmic mass is clearly anchored to the mouth of the exit tube, being only slightly and temporarily displaced by passing microscopic animals, and secondly because once the zoospores are fully formed they are clearly prevented for some time from escaping. In the example shown in Fig. 2 no vesicle wall could be seen, whilst in that illustrated in Fig. 3 a very thin wall was clearly visible. Dangeard figured no vesicle, but only a naked protoplasmic mass which later cleaved into zoospores. This failure to observe the vesicle is not surprising. He appears, however, to have been in error in supposing the zoospores to be uniflagellate.

It is to be remarked that the mode of zoospore formation is identical with that in *Pythium* and also in certain species of *Lagenidium*. However, although these two genera are normally classified in separate orders, the gap between them may really be a very small one.

In the infected *Lyngbya* which I examined zoospore production by the parasite was on a small scale and most specimens of the fungus produced resting spores rather than sporangia. Sometimes resting spores and zoosporangia were seen on the same thallus (Fig. 4).

Dangeard described resting-spore formation as the result of a sexual process and my observations tend to confirm this, although the process does not seem to be isogamous, but rather of the oogamous type described for *Lagenidium* and *Myzocytium*.

I have examined a very large number of specimens at all stages in the development of resting spores, and, although I have not actually seen the migration of the contents of the antheridium into the oogonium, it seems fairly clear that the resting spore is an oospore.

Fig. 4 illustrates the process. In the irregular elongated oogonium, an unmodified segment of the thallus, the contents become rounded off as an egg situated at one end of the oogonium. The cell adjoining this end is presumably the antheridium, but its contents do not aggregate until later, when, however, they clump against the wall partitioning the antheridium from the oogonium. The appearance is then of two oily protoplasmic masses side by side, and this was the appearance figured by Dangeard, although he failed

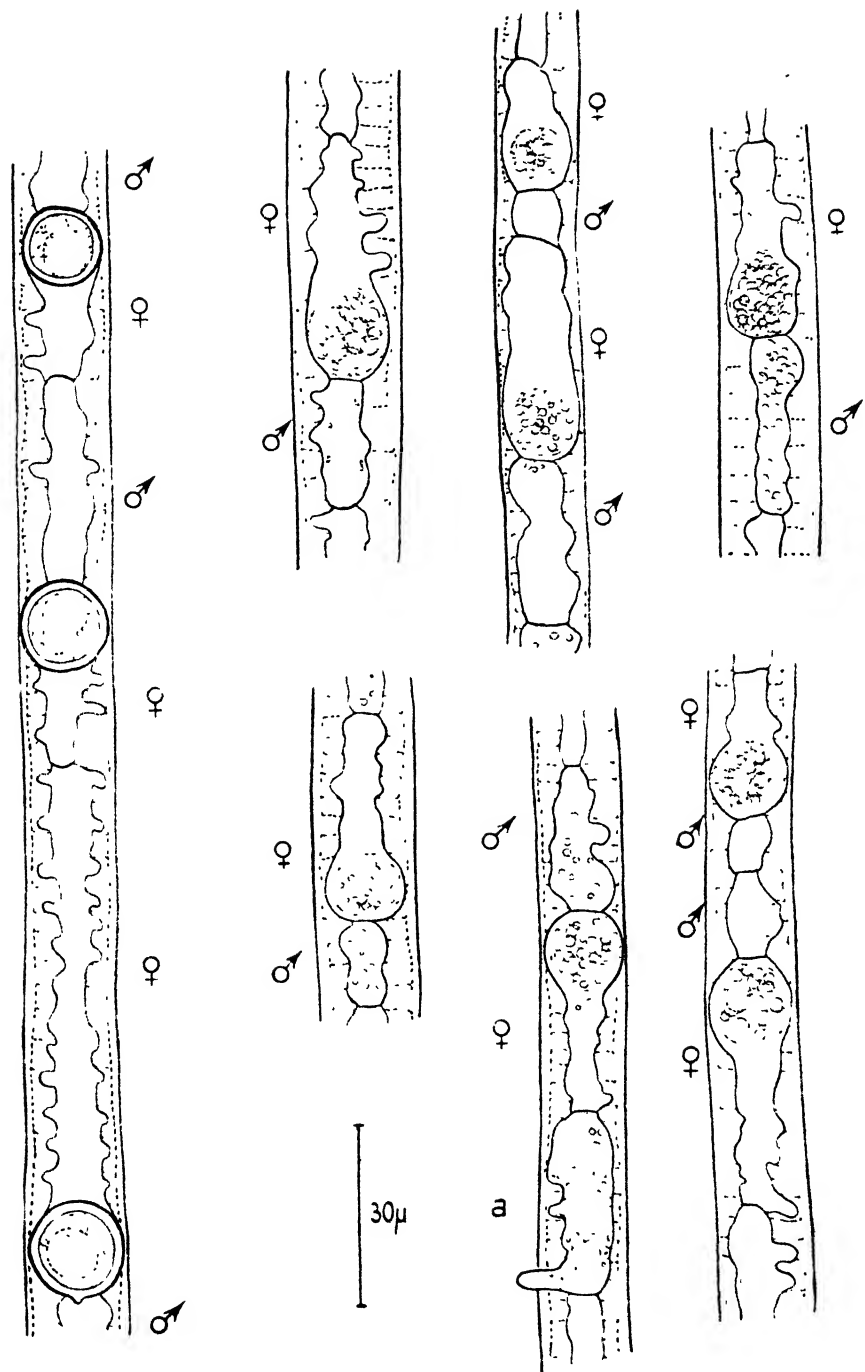


FIG. 4. *Lyngbya* sp. infected by *Resticularia nodosa* showing resting-spore formation. On the left is a thallus with three mature resting spores each with a smooth thick wall. Where the resting spore (oospore) is in contact with the oogonium wall the latter cannot be distinguished as a separate layer. The other drawings show early stages in resting-spore formation. The supposed sex of the segments of the thallus is indicated. At a a young zoosporangium is seen on the same thallus as a developing resting spore.

to observe the cross-walls. It seems that the contents of the antheridium migrate into the oogonium and fuse with the egg. However, there is no sign of a conjugation tube such as occurs in some species of *Lagenidium* and *Myzocyttium*, and I could not demonstrate a pore in the wall separating the oogonium with its oospore from the empty antheridium. This is due no doubt to the difficulty of observing the fungus through the sheathing dead cells of the host. The oospore surrounds itself with a thick, smooth, colourless wall. It contains one large oil globule usually accompanied by additional smaller ones.

Finally, in old material the thin cell-walls of both host and parasite disintegrate, probably as the result of bacterial action, and the resting spores are left within the resistant sheath of the host filament like beads in a length of glass tubing. Attempts to induce these spores to germinate have been unsuccessful.

SYSTEMATIC POSITION OF THE FUNGUS

From the description given above it will be seen that *Resticularia nodosa* agrees closely with certain genera of the Lagenidiales, especially *Lagenidium* and *Myzocyttium*. However, the inflation of the segments of the thallus which differentiates *Myzocyttium* from *Lagenidium* is not a feature of *R. nodosa*. There does not seem to be any justification for placing this fungus in a separate genus, and I therefore propose that the genus *Resticularia* should be abandoned and that the fungus described by Dangeard as the type of that genus should bear the name *Lagenidium nodosum* nov. comb. This is in keeping with the suggestion already made by Karling and is now justified by the new facts reported in this paper concerning the mode of formation of the resting spores.

It is perhaps worthy of note that the *Lyngbya* was infected by two other phycomycetes: *Rhizophidium megarrhizum* Sparrow, a parasite producing its epibiotic sporangia at the extremities of host filaments, and *Rhizophidium subangulosum* (Braun) Rabenh., which appeared to be entirely saprophytic and formed its epibiotic sporangia on the sides of dead *Lyngbya* filaments.

SUMMARY

The history of the genus *Resticularia* is considered and the unsatisfactory nature of our knowledge of species of the genus is stressed.

An account is given of a fungus parasitic on *Lyngbya* sp. which agrees in most particulars with Dangeard's original description of *R. nodosa*. The fungus is entirely intracellular, reproduces asexually by biflagellate zoospores, and forms resting spores by what seems to be a sexual process comparable with the oogamy of the Lagenidiales.

It is proposed to abolish the genus *Resticularia* and to transfer *R. nodosa* to the genus *Lagenidium* as *L. nodosum* nov. comb.

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A Petrified Example of *Alcicornopteris* (*A. Hallei* sp. nov.) from the Lower Carboniferous of Dunbartonshire

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With Plates XII and XIII

THE Clyde Plateau Lavas and the series of sedimentary rocks immediately below them form the major part of the Calciferosus Sandstone Series of the Lower Carboniferous Formation in the west of Scotland. In the sedimentary rocks below the lavas in the Kilpatrick Hills, Dunbartonshire, a large number of compressions and petrifications of plants have been found. Among the specimens collected were two nodules which came from a bed of sandstone consisting of quartz particles mixed with volcanic ash detritus. Both of these nodules showed on their upper surface examples of *Alcicornopteris* similar to some of those figured by Kidston (1924, Pl. CVIII, Figs. 3, 4, 5) under the specific name *A. convoluta*. One of these nodules on the surface of which the form of the plant was most clearly visible is shown in Pl. XII, Fig. 1. The other nodule on which the plant remains were less distinct was of a harder nature and there was evidence that it had petrified contents. It was cut into sections. This nodule, as will be demonstrated later, contained nothing but rachides (their anatomy reveals that they are not stems) and sporangia which formed apparently part of a single plant fragment. The rachides have similar dimensions and type of branching to those seen on the surface, and one cannot avoid coming to the conclusion that they are extensions of the parts shown on the surface and are therefore also to be referred to *Alcicornopteris*. It was found that the peel section method was not suitable for the preparation of sections from the nodule as the tissues are impregnated with silica and calcium carbonate. Some cells are completely filled with silica while neighbouring cells may contain calcite. This investigation is therefore based on four petrological sections cut parallel to the plan of bedding of the nodule, four petrological sections cut vertically to the bedding, and a few peel sections.

DESCRIPTION OF THE RACHIS SYSTEM

The sections exhibit numerous examples of rachides cut in various directions (Pl. XII, Figs. 2, 3), and it is clear that the thicker parts of the rachis system were on or near the upper surface of the nodule. The lesser branches extended downwards into the nodule where most of the small branches are found. A distinct impression is given that the system of branches was like a half-clenched hand and fingers and that the original plant fragment was

immature and not fully expanded from a state of veneration. This is supported by the state of the sporangia, which all appear full or nearly full of spores. No empty sporangia have been observed, although some must have lost spores, many of which are seen in the spaces between the sporangia, and most of the sporangia have breaks in their walls.

The thickest rachis is 3.4 mm. in diameter and the slenderest 0.43 mm. The epidermis of the rachides is poorly preserved and it is in only a few places that it can be seen distinctly. No evidence was found of hairs on the epidermis. The outer layers of the cortex (Pl. XII, Figs. 2, 3, Pl. XIII, Fig. 8) are usually partially disorganized and crushed and many of the cells have black contents. The rest of the cortex consists of thin-walled parenchyma (Pl. XII, Figs. 2, 4, Pl. XIII, Fig. 8). In some of the larger rachides groups or nests of thick-walled cells are found regularly spaced in the middle cortex (Pl. XII, Fig. 4). These sclerotic cells are more or less isodiametric and in peel sections their walls appear to be pitted.

THE VASCULAR STRANDS

Each branch of the rachis has a single vascular strand which forks at each forking of the rachis (Pl. XII, Figs. 5, 6). A single layer of cells (*e* in Pl. XII, Figs. 5, 6) with irregular and sometimes broken radial walls represents, no doubt, the sheath or endodermis. The fact that the radial walls appear to be different from the others suggests that a Casparian strip was present. One cannot be quite certain that the endodermis was complete as this peculiarity of the radial walls cannot be detected all round the bundle.

Inside the endodermis is a layer of thin-walled cells which have a thick deposit of mineral matter on their cell walls. It is likely that this layer represents the pericycle. An occasional thick-walled cell (*t* in Pl. XII, Figs. 5, 6) is found in this layer.

The xylem consists of a single strand of small tracheides. It is monarch with the protoxylem at the centre of the bundle. The tracheides when seen in longitudinal sections are seen to have fine closely coiled spiral thickenings. On the protoxylem side of the xylem is a group of small, thin-walled cells. If the bundle had possessed it this must represent the phloem, for there is nothing of the nature of phloem between the metaxylem and the endodermis on the other side of the xylem. If this interpretation of the structure of the bundle is correct, then we would be justified in assuming that the bundle is collateral and the xylem centripetal. This supports the view that these axes are rachides of a frond and are not stems or branches.

PEDICELS

Projecting abruptly from some of the small rachides we find still smaller appendages which are supplied with vascular tissue from the rachis bundle (Pl. XIII, Fig. 8). These slender appendages are here termed pedicels and they are about 0.2 mm. in diameter. The pedicel shown on the left in Pl. XIII, Fig. 10, is seen to have at least one tracheide in the centre surrounded by a

sheath of thin-walled tissue. Outside this is a layer of cells with dark contents, possibly the epidermis. This pedicel may be a branch of a larger one cut obliquely and seen on the right-hand side of Pl. XIII, Fig. 10.

Groups of pedicels are seen in several of the sections (e.g. Pl. XIII, Fig. 7), but it has not been possible to find out how they were arranged on the branches of the rachis system. The groupings, however, suggest that they formed little bunches either at the extremities or on the sides of the rachides.

THE SPORANGIA

Practically the entire space in the nodule not occupied by the rachides is closely packed with sporangia. In the peripheral layers of the nodule the sporangia are much crushed and distorted, but the cells of the sporangium walls and the spores are in a better state of preservation. In the more central parts the sporangia are not badly crushed (Pl. XII, Figs. 2, 3, 4) and an estimate of their diameter can be made. They were evidently about 0.7 mm. in breadth. The proximal ends can be distinguished by their attachment to pedicels, but their distal ends cannot be recognized. It is impossible, therefore, to get an estimate of their length. The sporangia were evidently considerably curved, for where the sporangium is not cut transversely (e.g. s_1 , Pl. XIII, Fig. 7 and s_7 , Pl. XIII, Fig. 9) we find that some of the wall cells are cut longitudinally and some transversely.

The wall of the sporangium consisted of a single layer of cells. Seen in transverse section (Pl. XIII, Fig. 11) the cells are all seen to have thick outer and anticlinal walls and thin inner walls. In longitudinal or tangential sections (Pl. XIII, Fig. 12) some of the wall cells are seen to be at least 0.5 mm. long, with pointed ends. The considerable length of the wall cells suggests that the sporangia were themselves long. There was evidently a tendency for the wall to split longitudinally. Most of the sporangia have more than one break in the wall. What may be a dehiscence slit is seen in section in the sporangium S_2 in the bottom right-hand corner of Pl. XIII, Fig. 7, and in the middle of the piece of sporangium wall shown in Pl. XIII, Fig. 11. There seems to have been only one type of cell in the wall of the sporangium except perhaps at the point where the wall is broken. At this point there is sometimes evident a cell of smaller diameter than the rest (Pl. XIII, Fig. 11, o). There is no evidence of a special annulus unless the whole wall is regarded as such.

The sporangia narrowed at the base. It is impossible to determine the nature of the apex of the sporangium. The longest section of a sporangium is 1.5 mm., but in all probability the complete sporangium was much longer; in fact the evidence provided by the sections is not inconsistent with the supposition that it might have been over 1 cm. in length like those of *Schuetzia bennieana* Kidston (1924, p. 425).

CONNEXION BETWEEN SPORANGIUM AND PEDICEL

Evidence that the pedicels bore sporangia has already been mentioned. The actual proof of the connexion is established by the evidence afforded by such

parts of the sections as those shown in Pl. XIII, Figs. 7 and 9. In Pl. XIII, Fig. 7, p_1 , in which under high magnification tracheides may be seen, connected with the tissue of the sporangium s_1 . In Pl. XIII, Fig. 9, pedicel p_7 is seen to be connected to sporangium s_7 and pedicel p_8 with sporangium s_8 . Other examples have been encountered in the sections and it is evident that the pedicels terminated in sporangia. There is some evidence too that the pedicels forked. We are therefore probably justified in believing that at or near the extremities of the branches of the rachis system small bunches of pedicels were attached. The sporangia were borne in small bunches and may have resembled the bundles of linear sporangia found in the genus *Schuetzia* or *Calathiops*.

THE SPORES

The spores in most of the sporangia are not well preserved (Pl. XIII, Figs. 7 and 9), and it is possible that they were immature and not completely cutinized. However, in some of the peripheral parts of the nodule they are very well preserved (Pl. XIII, Fig. 13). In the spores with the immature appearance the walls appear to be swollen and disorganized and mineralization phenomena simulate the appearance of cell contents. In a few sporangia some of the spores contain small, well-defined, spherical bodies of a constant size. It is possible that these are fungal reproductive structures, but no fungal hyphae have been seen.

The well-preserved spores (Pl. XIII, Fig. 13) appear to have a firm, dark-brown, well-cuticularized wall, which is covered with minute apiculations. The spores show a clear tetrad mark and many have split open along the tri-radiate ridge. In peel sections the layer of apiculations, probably representing perispore, is often separated from the rest of the wall so that objects which may be described as ghost spores are produced (Pl. XIII, Fig. 13). These show the outline of the spore and the fine granules which are the apiculations from the spore surface.

The apparently well-preserved spores in the outer part of the nodule vary in maximum diameter from $62\ \mu$ to $81\ \mu$ with an average maximum diameter of $65\ \mu$. Those in the inner parts of the nodule vary from $81\ \mu$ to $97\ \mu$, but as these appear to have been altered in other respects we are probably justified in assuming that the original dimensions ranged between $61\ \mu$ and $81\ \mu$, and that this fructification contained only one type of spore.

NOMENCLATURE

There is no doubt that these specimens from Dunbartonshire belong to the same genus as some of those which are included by Kidston (1887, p. 152) in the genus *Alaicornopteris*, which he defined as follows: 'Rachis ramifying by a series of dichotomies. Barren pinnae composed of foliaceous Rhacophyllum-like expansions. Fruiting portion consisting of much divided circinnately convoluted pinnae.' The type specimens which he figures (Kidston, 1887, Pl. VIII, Figs. 11-15) come from three different counties

and exhibit considerable differences. He assigns them all to *Alcicornopteris convoluta*. In 1924 Kidston (1924, p. 421) re-defines the genus and, assuming that *Alcicornopteris Zeilleri* Vaffier (1901, p. 124) belonged to the same genus, describes the fructification of the genus as synangial. This is, however, using the word in the wrong sense, for he writes that the sporangia are free. Vaffier refers to the presence of an indusium enclosing the fructifications. It is impossible to say more than that the fructifications appear to consist of bunches of linear structures which may be either linear sporangia, synangia, or indusial structures.

It would appear to be the better course for the present to disregard the actual fructifications of the genus and depend on the characters of the rachides. *Alcicornopteris* must be regarded simply as a form genus for a type of fructification rachis.

Kidston held the view that some of the specimens represented flat foliar expansions and that others with narrower branches bore the reproductive organs. The specimen in Pl. XII, Fig. 1, appears as a flattened structure and yet we have direct evidence from the petrified specimen that they were both originally cylindrical branches, and that the flat form is simply the result of compression.

A number of Lower Carboniferous plants are known, e.g. *Telangium bifidum* L. & H., *Anemites acadica* Daws. (1873, p. 26), *Diplopteridium teilianum* Walt. (1931, p. 352), in which the fronds consisted of a foliar part and a reproductive part. The foliar part had the characteristics of a fern frond, while the reproductive part consisted of a rachides which produced a fertile dichotomously branching system whose branches did not lie in one place.

There are good reasons for believing that the specimens included in *Alcicornopteris* by Kidston are all reproductive branches and are parts of fronds belonging to a number of different species and even genera. In *Calathiops* Göppert (1864, p. 268) authors have included a large number of forms of reproductive branches in most of which there is a tendency for dichotomy to occur in the smaller branches with a sympodial main rachis. In *Calathiops* some of the fructifications bore ovuliferous cupules, others bore bunches of linear structures similar to those of *Alcicornopteris Zeilleri* Vaffier. Gothan (1927, p. 10) has transferred *A. Zeilleri* to the genus *Calathiops*.

In the genus *Schuetzia* Geinitz the fructifications are described by Kidston (1924, p. 424) as campanulate pedicellate . . . composed of numerous curved lanceolate microsporangia, free except at their extreme base and borne on forking rachides without foliar expansions. He writes that *Alcicornopteris* and *Schuetzia* differ in that the 'synangia' (i.e. bunches of free sporangia) of *Alcicornopteris* are borne terminally on dichotomous branchlets, whereas in *Schuetzia* they are attached apparently spirally round the sides of an upright and undivided axis.

The sporangia of *Schuetzia* are about 1.25 cm. in length and 1 mm. in breadth, and contain spores with smooth walls, clearly defined triradiate ridge, and diameters ranging between 60 μ and 70 μ . There seems to be no

definite line of demarcation between *Alcornopteris*, *Calathiops*, and *Schuetzia*. The characteristics of the one grade into those of the other. The first was originally based on the nature of the rachis system, while the two other genera are based on the reproductive parts. They are ill-defined genera and must be regarded as of a provisional nature.

It has therefore been decided with some hesitation to include the fossil described in this paper in the genus *Alcornopteris* in view of the nature of the rachis system. It has, however, been given a distinctive specific name as it is the only example known in which we have definite data about the structure of the sporangia and spores. It is named *Alcornopteris Hallei* in recognition of the important additions Professor T. G. Halle has made to our knowledge of Pteridosperm fructifications.

Alcornopteris Hallei sp. nov.

Dichotomizing, terete, glabrous rachides ranging from 0.43 mm. to 3.4 mm. in diameter. Angle of dichotomy 90° or more. Cortex parenchymatous with sclerotic nests in the larger rachides. Vascular tissue consisting of one small monarch bundle of xylem (which is probably centripetal) and phloem. The bundle is probably collateral. Sporangia borne on the ends of slender pedicels which are attached in groups to the rachides. Sporangium about 0.7 mm. in diameter and over 2.8 mm. in length. Sporangium wall consisting of long prismatic cells with thick outer and radial walls. Spores between 61 μ and 81 μ in diameter with pronounced triradiate mark and finely apiculate rounded surface.

Type specimen in sections 395-407 Figured Slide Collection, Dept. of Botany, University of Glasgow.

From sandstone exposed above small waterfall in tributary entering the south side of the Loch Humphrey Burn, Kilpatrick Hills, Dunbartonshire, Scotland.

Horizon. Below the Clyde Plateau Lavas. Calciferous Sandstone Series. Lower Carboniferous.

ATTRIBUTION TO PTERIDOSPERMAE

The sporangium of *Alcornopteris Hallei* appears to have no differentiated annulus; all the wall cells are of the same type. This is not in itself a criterion by which we may distinguish it from fern sporangia, for some of the primitive ferns, e.g. *Stauropteris*, and many living families, e.g. Marsiliaceae, Salviniaceae, and Marattiaceae, include types with exannulate sporangia. The Marsiliaceae and Salviniaceae are probably specialized types and there is evidence in support of the view that they have evolved from annulate ancestors. The Marattiaceae are synangial, yet some of them, e.g. *Angiopteris*, have special indurated cells in the sporangium wall.

The most clearly defined difference between the sporangia of *Alcornopteris Hallei* and the ferns lies in the nature of the wall cells which are indurated on their *outer* and radial walls, while in the ferns the induration is always on the

inner and radial walls of the cells of the annulus. We must suppose that walls of the sporangia of *A. Hallei* when subjected to dry conditions curled inwards. In preliminary stages of fossilization the sporangia were probably saturated with water, for there is no sign of shrivelling, and they are presumably seen in their relaxed condition in the fossil.

The simple type of dichotomy of the rachis without aphlebiae or other foliar expansions borne in relation to the branching has been observed in many Lower Carboniferous plants many of which are in all probability seed plants.

The type of vascular supply found in what have been called rachides bears a close resemblance to that of a foliar bundle and supports the view that these cylindrical branches are rachides or parts of a leaf or frond. In details of structure the bundle bears a close resemblance to the bundles found in the leaf-trace system of *Medullosa* (Scott, 1889, Pl. VII, Figs. 14, 15); for if the interpretation of the structure of the bundle given on p. 446 is correct, the xylem is entirely centripetal and the bundle itself is a collateral bundle. This type of bundle does not suggest an affinity with the ferns.

Taking all these points into consideration it seems reasonable to conclude that this fossil is part of a Pteridosperm.

SUMMARY AND CONCLUSIONS

Alcicornopteris Hallei is the first example of a fairly complete microsporangiate or polliniferous fructification of a pteridosperm found in the petrified state.

Its attribution to the Pteridospermae is based on the exannulate type of sporangium, the position of the thickening on the walls of the sporangium cells, the presence of sclerotic nests in the rachides, and the structure of the vascular strands.

The structure of the branching axes indicated that they were parts of a frond and not stems.

The spores bear a closer resemblance to those of Pteridophyta than to pollen of pteridosperms and gymnosperms, and it is probable that the plant was not siphonogamous.

In comparison with the synangial types of pollen-bearing organs of the Upper Carboniferous *Alcicornopteris Hallei* seems relatively simple and may possibly be regarded as occupying an intermediate position between the type of organization found in the Upper Devonian *Archaeopteris* and the Upper Carboniferous synangial types. It might represent a precursor of the *Dolerotheca* or *Potoniaea* synangia (Halle, 1933, pp. 44, 72) in which a large number of linear sporangia are aggregated together. There is no obvious relationship between it and the Upper Carboniferous cyclic synangial types, e.g. *Whittleseya* and *Aulacotheca*.

The author acknowledges with thanks a grant towards the cost of the illustrations awarded by the Carnegie Trust for the Universities of Scotland.

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PLATES XII and XIII

Illustrating J. Walton's article on 'A Petrified Example of *Alccornopteris (A. Hallei sp. nov.)* from the Lower Carboniferous of Dunbartonshire'.

All illustrations are from untouched photographs.

Key to lettering on the figures: *e*, endodermis; *o*, break in wall of sporangium; *p*, pedicel; *ph*, phloem; *s*, sporangium; *sc*, sclerotic nests; *t*, thick-walled cells; *v*, vascular strand; *x*, xylem.

F.S.C. Figured Slide Collection, Dept. of Botany, University of Glasgow.

Alccornopteris Hallei sp. nov.

Fig. 1. Compression in the bedding plane on the surface of a nodule. Natural size. Hunterian Museum, Univ. Glasgow. Pb 2344.

Fig. 2. Petrological section showing a rachis with the characteristic wide-angled dichotomies, sections of several smaller rachis branches, and numerous sporangia. ($\times 9.3$.) F.S.C. 396.

Fig. 3. Petrological section showing some of the smaller rachides cut near dichotomies and numerous sporangia. ($\times 8.8$.) F.S.C. 400.

Fig. 4. Peel section of a nodule showing one of the major rachides cut transversely just above a dichotomy and the dichotomies of the two branches. ($\times 8.8$.) F.S.C. 407.

Fig. 5. Part of the cortex and the vascular bundle of a medium-sized rachis. ($\times 143$.) F.S.C. 398.

Fig. 6. Vascular strand just above a dichotomy. ($\times 159$.) F.S.C. 399.

Fig. 7. Parts of a number of sporangia grouped round some pedicels. ($\times 46.5$.) F.S.C. 398.

Fig. 8. Section near the tip of a small rachis showing the attachment of two pedicels. ($\times 46.5$.) F.S.C. 400.

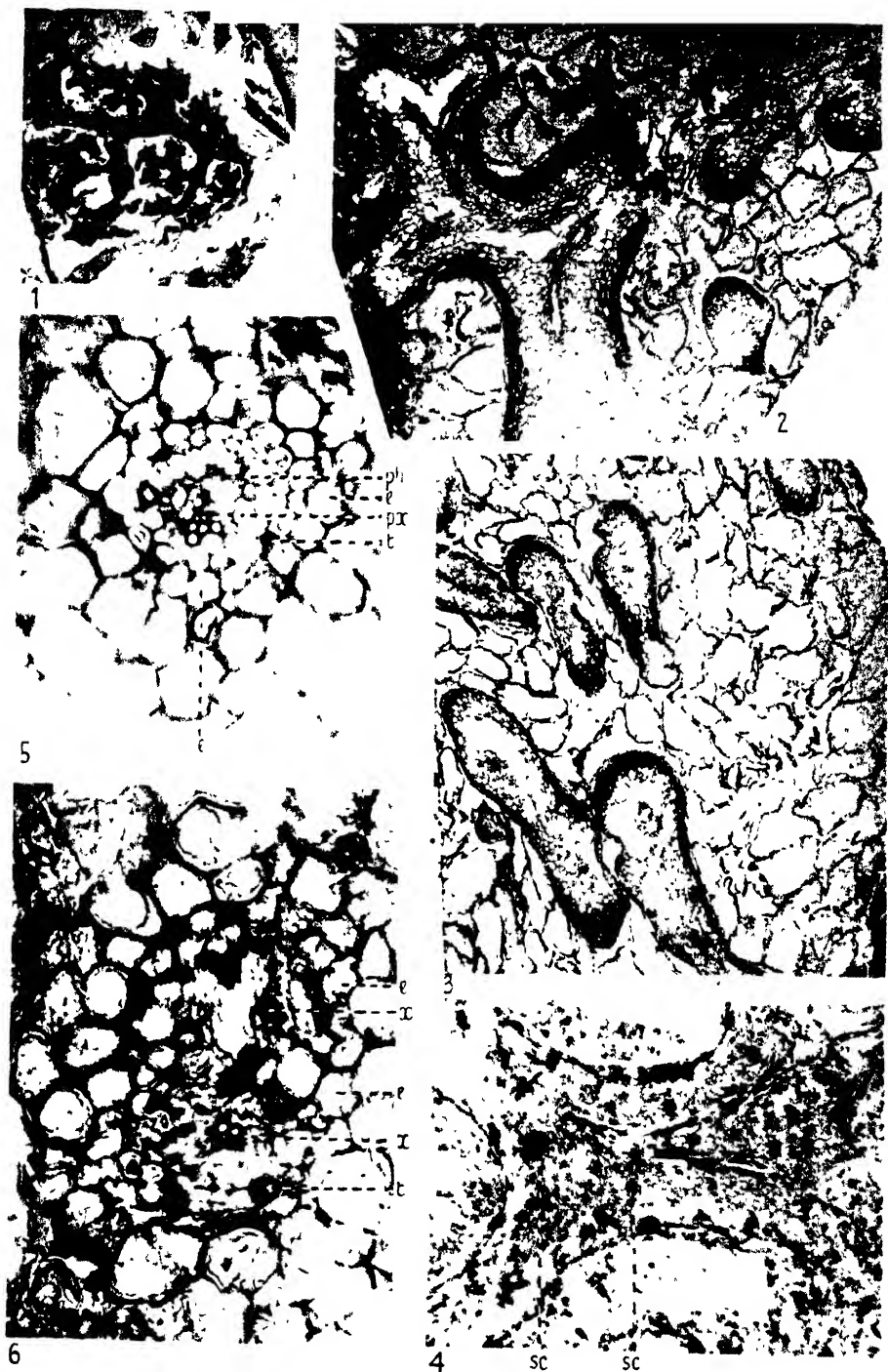
Fig. 9. Part of a section showing the connexion between pedicels and sporangia. *p*₇, in which tracheides are present, is connected to sporangium *s*₇, the wall of which is cut tangentially. *p*₈ is continuous with the base of sporangium *s*₈. ($\times 81$.) F.S.C. 398.

Fig. 10. Part of a curved or forked pedicel (*p*₄ in Fig. 7 with a xylem strand cut transversely on the left and on the right. ($\times 140$.) F.S.C. 398.

Fig. 11. Transverse section of part of a sporangium wall. The break at *o* may represent line of dehiscence. ($\times 140$.) F.S.C. 398.

Fig. 12. Tangential section through the wall of a sporangium showing the long wall cells with pointed ends. ($\times 140$.) F.S.C. 398.

Fig. 13. Spores in the cavity of a sporangium in a peel section. Complete spores are seen with triradial mark where spore has opened. The 'ghosts' are superficial apiculations on spores which have been torn off in making the peel section. ($\times 140$.) F.S.C. 403.





J. WALTON — *Alcacornopteris*

Physiological and Ecological Studies in the Analysis of Plant Environment

IV. The Interaction between Light Intensity and Mineral Nutrient Supply on the Uptake of Nutrients by the Bluebell (*Scilla non-scripta*)

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With fifteen Figures in the Text

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INTRODUCTION

IN a detailed analysis of the autecology of the bluebell (*Scilla non-scripta*), three papers have already been published on various aspects of the general problem. Firstly, multiple regressions linking the distribution of the bluebell with the degree of shading have been derived for a number of woodland communities, and these have demonstrated a precise correlation between the two variables (Blackman and Rutter, 1946). Secondly, the effects on growth and development of varying light intensity, coupled with the influences of nitrogen, phosphorus, and potassium, alone and in combination, have been simultaneously investigated in field experiments and under woodland conditions. It has been found that whereas the growth of the bluebell is little affected by

the level of mineral nutrition, it is especially sensitive to a reduction in the light intensity (Blackman and Rutter, 1947). This sensitivity is largely due to the effects of light intensity on the net assimilation rate, since it has been established that the assimilation rate is *directly proportional* to the logarithm of the light intensity (Blackman and Rutter, 1948). Below 0.6 of daylight the fall in assimilation rate is no longer offset by the influence of shading in increasing the total leaf area per plant, and so the growth rate is reduced. Moreover, growth ceases when the compensation point is reached at 0.1 of daylight.

This, the fourth paper, is concerned with the effects of light intensity and nutrient supply on the seasonal uptake of nitrogen, phosphorus, and potassium and the subsequent distribution of these elements in the bulb, leaves, inflorescence, and seed. The data have been derived from two of the largest field experiments—namely, expt. III, 1938, and expt. IV, 1939. The design and general experimental procedure have already been given in the second paper (Blackman and Rutter, 1947). Briefly, in both years graded flowering bulbs were planted in plots in the autumns of 1937 and 1938 and received early in the following springs combinations of nitrogen (at 50 lb. nitrogen per acre), phosphorus, and potassium (each at 75 lb. per acre), viz. C, N, P, K, NP, NK, PK, and NPK. Superimposed on these eight nutrient treatments there were three levels of light intensity, making in all twenty-four treatments. One of the light treatments was full daylight and the lower light intensities were achieved by placing on the plots wooden frames covered either with butter-muslin, gauze, or perforated zinc.

Samples of plants were withdrawn from time to time and after being split up into bulb plus roots, leaves, and inflorescence, the parts were dried in an oven at 100° C. and weighed.

The effects of the experimental treatments on the weight changes of the whole plant and its parts have been discussed in the second paper; the effects on mineral uptake have been derived by combining these weight data with the corresponding percentage contents of the samples obtained in the two experiments.

EXPERIMENTAL PROCEDURE

Chemical analytical methods

Grinding technique. The dried bulbs were very finely ground in an end-runner mill and the leaves and flowers in a Christie-Norris micro-mill. As the season advanced, the flower stalks became progressively more fibrous and in consequence more difficult to grind, so the samples were made to pass the finest mesh possible. The quantities of some of the initial samples were very small; in consequence they were ground in a Wiley micro-mill from which there is almost complete recovery.

Estimation of nitrogen. Total nitrogen was determined by the Kjeldahl method. About 0.5 g. of material was digested with 5 g. anhydrous Na_2SO_4 , 3 g. $\text{Na}_2\text{S}_2\text{O}_7$, 0.5 g. salicylic acid, 0.1 g. selenium, and 15 to 20 c.c. concen-

trated nitrogen-free sulphuric acid. The use of selenium as a catalyst has been discussed by Ashton (1936, 1937), on whose advice the foregoing quantities were used. The mixture cleared in about 30 minutes and was allowed to digest for a further hour. After cooling, the contents of the digestion flasks were made up to 100 c.c. with water, and 5-c.c. aliquots were distilled in the micro-Kjeldahl distillation apparatus of Pregl (1924).

Estimation of phosphorus. Determinations of phosphorus were made colorimetrically by the method of Fiske and Subbarow, as described by Greenhill and Pollard (1935).

Estimation of potassium. For potassium determinations, a modification of the method of Ismail and Harwood (1937) was used. In essence the method is to precipitate potassium from solution as potassium silver cobaltinitrite by the addition of silver nitrate and sodium cobaltinitrite in the presence of acetone. The precipitate was thrown down in a centrifuge, washed, and dissolved in nitric acid; the silver in the potassium silver cobaltinitrite complex was then titrated against 0.01 normal ammonium thiocyanate. This complex is of variable chemical composition, but if it is precipitated under the standard conditions described by Ismail and Harwood, the amount in mg. of potassium present should be obtained by multiplying the number of c.c. of ammonium thiocyanate used by the factor 0.4878.

In spite, however, of careful standardization of conditions, including the temperature at which the precipitate was formed and washed, it was not found possible to obtain consistent results when testing the method by the analysis of potassium sulphate solutions of known concentration.

For the purposes of the phosphorus analyses, the procedure involved taking up the washed plant material in 10 normal sulphuric acid and then diluting to 1 normal, and it seemed rational to use an aliquot of this solution for potassium analyses after its neutralization by 2 normal caustic soda. In effect this technique meant that the potassium which was derived from the plant material was contained in an almost saturated solution of sodium sulphate.

It was observed that when the precipitate derived from the standard potassium sulphate solution was stirred up in acetone it was sticky and lumpy in consistency and could not be finely broken up in the process of washing. On the other hand, precipitates obtained from the ash solution could be evenly distributed through the washing medium. The difference between the media from which the precipitates were thrown down was suspected as the cause of the error, and consequently known amounts of potassium sulphate were dissolved in the sodium sulphate solution obtained by the means already described, viz. the neutralization of 1N sulphuric acid by 2N caustic soda. In this way a fine easily dispersable precipitate was obtained, and the method now gave consistent results. It was concluded, therefore, that the previous variable results were due to the difficulty of washing out inclusions of silver nitrate from the precipitate, and that this difficulty had been overcome by the new precipitation technique.

It would be expected that the composition of the precipitate, and in

consequence the conversion factor, would be affected by the alteration in technique. In order to determine the new factor, twelve solutions of known strength of potassium sulphate in sodium sulphate solution were analysed. The first two columns of Table I show the known weight of potassium used and the corresponding thiocyanate titration. From these figures the following regression was constructed:

$$y = 2.161x + 0.15 \text{ (S.E. of } b = 0.045),$$

where y is the titration in c.c. thiocyanate and x the weight in mg. of potassium taken. It is concluded that the quantity 0.15 represents a constant amount of sodium silver cobaltinitrite, thrown down on account of the very high concentration of sodium present: that such a precipitate could be formed was confirmed by experiment.

TABLE I

Determination of Conversion Factor in the Estimation of Potassium

Mg. K = x .	c.c. thiocyanate = y .	mg. K recovered.	Percentage error.
0.223	0.62	0.217	-2.7
0.319	0.86	0.329	+3.1
0.427	1.07	0.426	-0.2
0.621	1.56	0.652	+5.0
0.703	1.70	0.717	+2.0
0.816	1.90	0.810	-0.7
0.366	0.94	0.366	0.0
0.173	0.50	0.162	-6.4
0.417	1.05	0.416	-0.2
0.680	1.60	0.671	-1.2
0.616	1.48	0.615	-0.2
0.830	1.90	0.810	-2.4

After the subtraction of this constant from the titration, the amount of potassium is found by multiplying by $\frac{1}{2.161} = 0.4627$. The recovery of potassium as given by this formula is seen in column 3 of Table I and the error, as a percentage of the amount taken, in column 4.

Statistical treatment of data

Grouping of samples. On account of the large number of samples to be analysed, samples from duplicate plots were bulked, but in most instances separate analyses were made of bulb, leaf, and flower in every treatment at every sampling occasion. Because the quantities were small during the initial stages of growth, the leaf and flower were bulked together for each treatment for the first two occasions in expt. III, 1938, and in expt. IV, 1939, the corresponding manurial treatments at each light intensity were bulked together for the initial sample since the screens were only subsequently placed on the plots.

As the total weights of each sample were also known, besides the percentage content of each element, the total amounts of the elements present could be derived. As a result these figures in aggregate amounted to several thousand

and it has not been feasible to carry out a statistical analysis in every instance. Analyses of variance were undertaken, in the first place, on the percentage content and the total amount in the whole plant of each of the three elements. Since the greater part of the plant is bulb material, and since the figures for the composition of plant and bulb were similar, separate statistical consideration has not in general been given to the latter. Further analyses of variance, however, were calculated for the percentage contents of the leaf, since they often differed markedly from those of the other parts of the plant.

Method of Statistical analyses. Statistical treatment of the total amounts of each element was carried out without transformation. But as the percentage contents of each element were all less than 15 per cent., the figures were first converted to the angular scale of θ , where $\sin^2 \theta = P$, the percentage value (Bliss, 1937 and 1938).

As the screens in 1938 were not placed on the plots until after the first sample, these initial data have not been included in the analysis of the effects of light during the course of seasonal growth. Similarly in 1939, when both sampling and chemical analysis were on a restricted scale for the first sample, the figures have not been included in the statistical analyses.

The distribution of the error between the several sampling occasions was examined by the LI test (Nayer, 1936) and the values of LI obtained are given in Table II. These values which do not reach the required level ($P = 0.05$) are marked with an asterisk; they are mg. nitrogen and phosphorus per plant in 1938, and mg. potassium per plant in 1939.

TABLE II

Experiments III, 1938, and IV, 1939. Values of LI for Errors of Sampling Occasions in the Chemical Analyses of the Whole Plant

	Nitrogen.	Phosphorus (P_2O_5).	Potassium.
Per cent. content, 1938	0.879	0.886	0.778
„ „ 1939	0.760	0.919	0.906
Mg. per plant, 1938	0.579*	0.652*	0.867
„ „ 1939	0.934	0.910	0.600*

* Significance level $P < 0.05$.

Since the lack of balance in the distribution of the error does not affect the analysis of variance where it applies to comparisons involving only a *single* degree of freedom, these three sets of 'unbalanced' data were dealt with by grouping the sampling occasions into two periods; i.e. in 1938 the mean values of samples I, II, and III (period I) were compared with the means of samples IV and V (period II), and in 1939 sample II was compared with the means of samples III and IV.

In both 1938 and 1939 statistical treatment of the leaf and inflorescence data was restricted to two sampling occasions only, and consequently the question of the balance of error between occasions does not arise.

EXPERIMENTAL RESULTS

Seasonal course of nutrient uptake

The changes with time in the total amount of nitrogen, phosphorus, and potassium present in the plant together with the changes in total plant weight during the growing season are shown in Figs. 1 and 2. In order to obtain direct comparisons of the seasonal drift, the changes in each element are expressed as ratios of the amounts present in the initial sample, i.e. when active growth commenced in the spring.

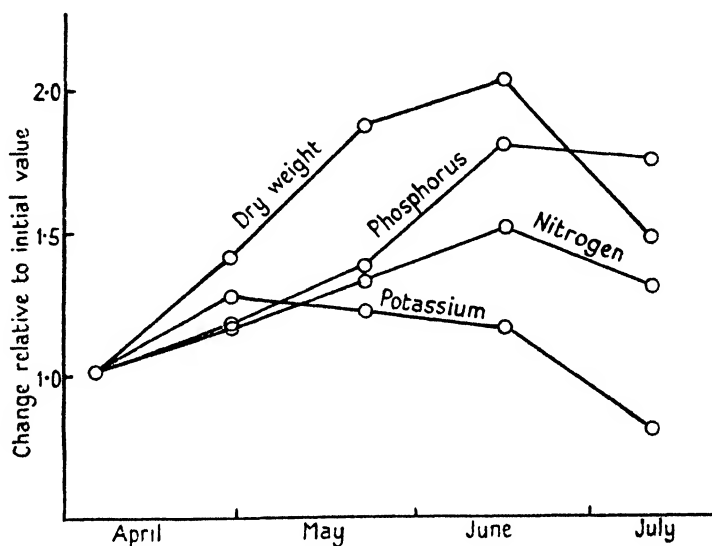


FIG. 1. Comparative seasonal changes in dry weight and the uptake per plant of nitrogen, phosphorus, and potassium. Changes expressed as ratios of amounts present in initial sample (expt. III, 1938).

It is clear that in both years the course of uptake is dissimilar for each of the elements. It is equally apparent that the trends in nutrient uptake do not follow precisely the changes in plant weight. In the case of potassium a maximum is reached by the time the plants have commenced flowering and this peak is followed by a progressive decline. On the other hand, for nitrogen and phosphorus, uptake proceeds steadily until flowering has ceased and there is no loss of either phosphorus or nitrogen until the onset of leaf senescence. Thus in expt. III, by June 15 the leaves were beginning to die back and only subsequently does the amount of phosphorus and nitrogen decline. In the 1939 experiment (Fig. 2) this phase had not been reached when the sample on July 12 was taken.

Whereas the losses of nitrogen and phosphorus can be ascribed to leaf senescence, such an explanation cannot be advanced for potassium as the leaves were still green when the level of potassium started to fall. Wallace (1930) has found that a great proportion of the potassium present in the

leaves of fruit-trees is removed by immersion in water and the most likely explanation of the present observation is that rain differentially leaches out potassium from the bluebell leaves.

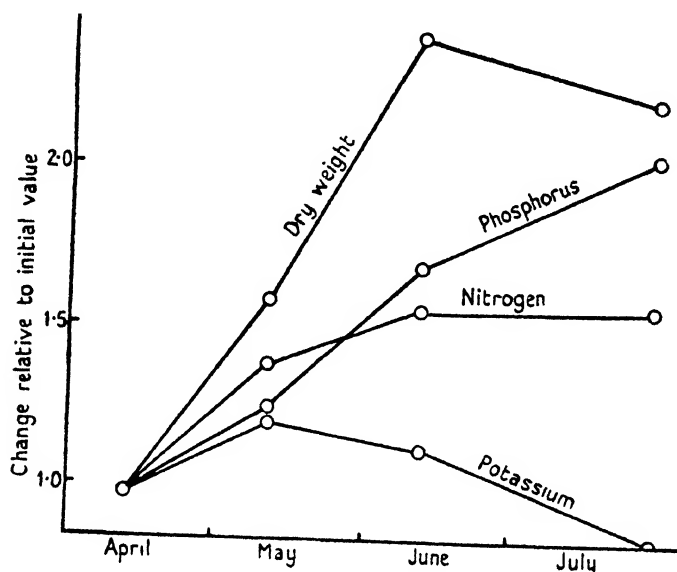


FIG. 2. Comparative seasonal changes in dry weight and the uptake per plant of nitrogen, phosphorus, and potassium. Changes expressed as ratios of amounts present in initial sample (expt. IV, 1939).

This supposition is lent further support by the relative changes in the amounts of the three elements present in the leaves in the later half of the growing period, see Tables III and IV. The fall in the potassium content of the leaves is much sharper than the corresponding reductions in phosphorus and nitrogen.

TABLE III

Experiment III, 1938. The Uptake and Distribution in the Plant of Nitrogen, Phosphorus, and Potassium, during the Growing Season

	Amount per Plant in Milligrams						
	Date of sampling occasion.						
	Oct. 1937.	I Apr. 4.	II Apr. 29.	III May 22.	IV June 15.	V July 11.	Sig. diff. ($P=0.05$).
<i>Nitrogen</i>							
Bulb	20.35	12.70	11.37	15.99	23.78	28.11	—
Leaf	—	9.15	13.78	7.97	3.13	—	—
Flower	—			4.95	6.07	—	—
Whole plant	20.35	21.85	25.15	28.91	32.98	28.11	—
Whole plant (period means)			25.30		30.55		1.39

TABLE III (cont.)

Amount per Plant in Milligrams

Date of sampling occasion.

	Oct. 1937.	I Apr. 4.	II Apr. 29.	III May 22.	IV June 15.	V July 11.	Sig. diff. ($P=0.05$).
<i>Phosphorus</i> (P_2O_5)							
Bulb	5.62	4.14	4.01	5.55	8.30	10.89	—
Leaf	—	2.16	3.29	1.50	0.59	—	—
Flower	—			1.57	2.31	—	—
Whole plant	5.62	6.30	7.30	8.62	11.20	10.89	—
Whole plant (period means)			7.41		9.14		0.54
<i>Potassium</i>							
Bulb	12.51	8.43	7.52	7.61	9.95	10.98	—
Leaf	—	5.07	9.65	4.24	1.31	—	—
Flower	—			4.72	4.35	—	—
Whole plant	12.51	13.50	17.17	16.57	15.61	10.98	0.85

TABLE IV

Experiment IV, 1939. The Uptake and Distribution in the Plant of Nitrogen, Phosphorus, and Potassium during the Growing Season

Amount per Plant in Milligrams

Date of sampling occasion.

	Oct. 1938.	I Apr. 12.	II May 11.	III June 12.	IV July 31.	Sig. diff. occs. II-IV
<i>Nitrogen</i>						
Bulb	30.93	34.50	28.58	52.41	80.58	—
Leaf	—	15.55	31.95	15.08	—	—
Flower	—	—	10.11	11.19	—	—
Whole plant	30.93	50.05	70.64	78.68	80.58	4.78
<i>Phosphorus</i> (P_2O_5)						
Bulb	13.11	12.42	9.98	20.17	32.75	—
Leaf	—	3.46	6.58	3.14	—	—
Flower	—	—	3.58	3.69	—	—
Whole plant	13.11	15.88	20.14	27.00	32.75	1.36
<i>Potassium</i>						
Bulb	23.53	24.54	13.21	22.90	28.90	—
Leaf	—	9.17	21.60	8.04	—	—
Flower	—	—	6.35	7.18	—	—
Whole plant	23.53	33.71	41.16	38.12	28.90	—
Whole plant (period means)			41.16	33.51		0.83

Reference to Tables III and IV also shows that the rate of salt uptake during the winter months varies between the two experiments. Over the period of October to April there was little increase in nitrogen, phosphorus,

or potassium in expt. III (Table III) but considerable increases in expt. IV (Table IV). Moreover, in expt. IV the winter accumulation of nitrogen is greater than that of potassium, while for phosphorus there has been a far slower rate of absorption.

TABLE V

Experiment III, 1938. The Seasonal Variation in the Percentage Content of Nitrogen, Phosphorus, and Potassium

Percentage Content of Dry Weight							
	Date of sampling.						Sig. diff. ($P=0.05$).
	Oct. 1937.	I Apr. 4.	II Apr. 29.	III May 22.	IV June 15.	V July 11.	
<i>Nitrogen</i>							
Bulb	1.92	1.41	0.98	1.03	1.34	1.81	—
Leaf	—	{ 4.21	3.53 }	3.70	2.83*	—	—
Flower	—			1.90	1.71	—	—
Whole plant	1.92	1.96	1.61	1.42	1.48	1.81	—
Whole plant (angular scale)	—	8.03	7.27	6.84	6.96	7.69	0.15
<i>Phosphorus (P₂O₅)</i>							
Bulb	0.53	0.46	0.34	0.35	0.47	0.69	—
Leaf	—	{ 0.99	0.84 }	0.70	0.59*	—	—
Flower	—			0.62	0.63	—	—
Whole plant	0.53	0.56	0.46	0.42	0.50	0.69	—
Whole plant (angular scale)	—	4.29	3.90	3.72	4.06	4.75	0.12
<i>Potassium</i>							
Bulb	1.18	0.94	0.64	0.49	0.58	0.72	—
Leaf	—	{ 2.32	2.45 }	1.95	1.25*	—	—
Flower	—			1.81	1.23	—	—
Whole plant	1.18	1.20	1.10	0.83	0.72	0.72	—
Whole plant (angular scale)	—	6.28	5.99	5.19	4.83	4.80	0.15

* When these data are transformed to the angular scale, the differences between percentage contents of leaf on May 22 and June 15 are in each case significant.

Once active growth starts, there is at first a transference of all three elements from the bulb to the leaves. Subsequently this transference is reversed. This reversal of translocation is also reflected in the changes in percentage content shown in Tables V and VI. The total nitrogen, phosphorus, and potassium contents of the leaves—and incidentally the dry weights—attain their maxima together, if it is assumed that in the first 1938 samples when the leaf and

inflorescence were bulked together the relative contents were of the same order as in later samples. After reaching peaks on April 29, 1938, and May 11, 1939, the contents of each element fall, slightly at first, and then more rapidly as the leaves die back.

TABLE VI

Experiment IV, 1939. The Seasonal Variation in the Percentage Content of Nitrogen, Phosphorus, and Potassium

	Percentage Content of Dry Weight					
	Date of sampling occasion.					
	Oct. 1938.	I Apr. 12.	II May 11.	III June 12.	IV July 31.	Sig. diff. ($P=0.05$).
<i>Nitrogen</i>						
Bulb	0.92	1.62	1.07	1.13	1.52	—
Leaf	—	4.42	4.14	2.86*	—	—
Flower	—	—	2.72	1.85	—	—
Whole plant	0.92	2.04	1.81	1.37	1.52	—
Whole plant (angular scale)	—	—	7.69	6.68	7.04	0.18
<i>Phosphorus (P₂O₅)</i>						
Bulb	0.39	0.59	0.37	0.44	0.61	—
Leaf	—	0.97	0.86	0.61*	—	—
Flower	—	—	0.96	0.61	—	—
Whole plant	0.39	0.64	0.52	0.47	0.61	—
Whole plant (angular scale)	—	—	4.09	3.90	4.45	0.10
<i>Potassium</i>						
Bulb	0.70	1.14	0.49	0.50	0.54	—
Leaf	—	2.60	2.78	1.73*	—	—
Flower	—	—	1.70	1.19	—	—
Whole plant	0.70	1.37	1.06	0.69	0.54	—
Whole plant (angular scale)	—	—	5.82	4.64	4.15	0.16

* When these data are transformed to the angular scale all differences between percentage contents of leaf are significant.

Apart, however, from the transference of material to the bulb from the leaves there is also the question of the mineral nutrients lost in the dead capsules and seed. The absolute amount of each element in the inflorescence increases until the one from last occasion; that is, before the seed has been shed and the final sample taken. In expt. IV more than half of the nitrogen and phosphorus found in the flower on June 12 reappeared in the seed, although only one-tenth of the potassium remained (see Table XVII).

The effects of varying light intensity on mineral nutrient uptake

The main effects of light intensity on the seasonal uptake of the three elements can be seen in Figs. 3 and 4 and Tables VII, VIII, and IX. In the

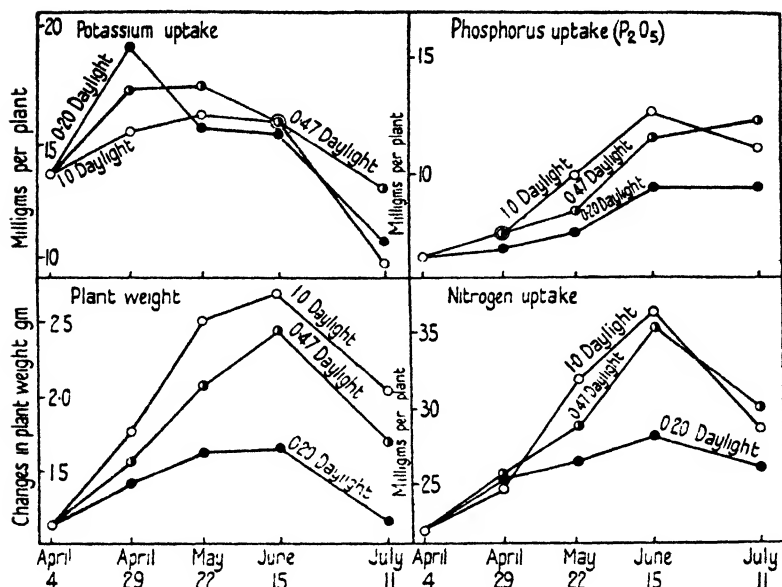


FIG. 3. The effects of varying light intensity on both the changes in dry weight and the uptake of nitrogen, phosphorus, and potassium during the period of seasonal growth (expt. III, 1938).

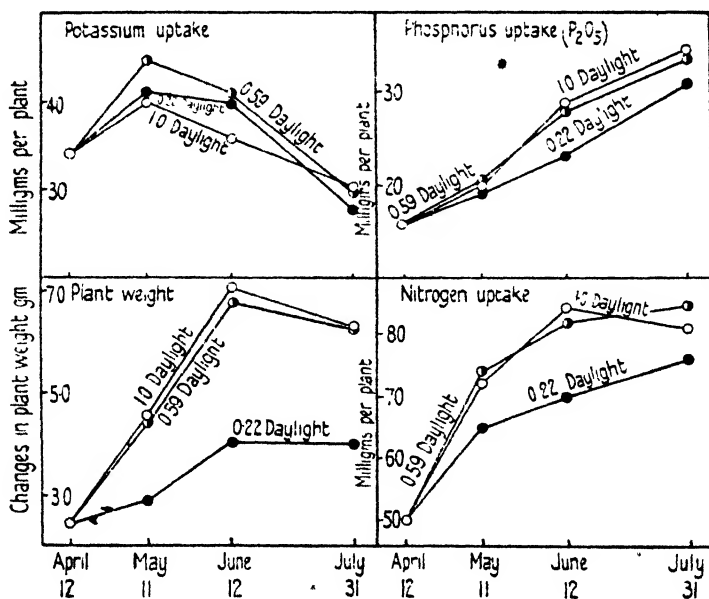


FIG. 4. The effects of varying light intensity on both the changes in dry weight and the uptake of nitrogen, phosphorus, and potassium during the period of seasonal growth (expt. IV, 1938).

case of both nitrogen and phosphorus, light intensity decreases the total uptake, but this reduction is not marked until the intensity has reached less than about two-thirds daylight, for in no instance is there any significant difference between the values for daylight and 0.59 or 0.47 of daylight (Tables VII and VIII).

TABLE VII

Experiment III, 1938. The Effects of Varying Light Intensity on the Seasonal Uptake of Nitrogen and Phosphorus

	Uptake (mg. per plant)		
	Date of sampling.		
	Period A, (Samples I-III).	Period B, (Samples IV-V).	Whole season.
<i>Nitrogen</i>			
1.0 daylight*	26.07	32.27	28.47
0.47 ,,	24.98	32.42	27.92
0.20 ,,	24.85	26.96	25.63
Sig. diff. ($P = 0.05$)		2.41	2.47
<i>Phosphorus</i> (P_2O_5)			
1.0 daylight	7.83	11.85	9.44
0.47 ,,	7.34	12.03	9.21
0.20 ,,	7.05	9.26	7.94
Sig. diff. ($P = 0.05$)		0.93	0.29

* The light intensities given in this and subsequent tables for expt. III are the mean intensities during the season. During the course of the experiment there were some small changes in the light transmission of the screens since the materials faded under the action of sunlight and rain.

TABLE VIII

Experiment IV, 1939. The Effects of Varying Light Intensity on the Seasonal Uptake of Nitrogen, Phosphorus, and Potassium

Light intensity.	Uptake (mean mg. per plant for all samples)		
	Nitrogen.	Phosphorus. (P_2O_5).	Potassium.
1.0 daylight	79.24	27.82	34.76
0.59 "	80.21	27.76	37.57
0.22 "	70.45	24.31	35.84
Sig. diff. ($P = 0.05$)	6.66	1.68	not sig.

In contrast to nitrogen and phosphorus, uptake of potassium reaches a maximum at 0.47-0.59 of daylight and then falls off at 0.20-0.22 of daylight to about the same level as in full daylight. In 1939 the differences observed are not significant (Table VIII), but in 1938 the values for 0.47 daylight are in the main significantly higher than for either the higher or lower level of daylight (Table IX).

In the analyses of variance there are significant interactions of light with sampling occasion, for the total uptake of all three elements in 1938, but not in 1939. With nitrogen and phosphorus, the interaction in 1938 is due to the absence of significant differences between the values at the three light levels

TABLE IX

Experiment III, 1938. The Effects of Varying Light Intensity on the Seasonal Uptake of Potassium

Light intensity.	Date of sampling.				Mean.
	Apr. 29.	May 22.	June 15.	July 11.	
1.0 daylight	15.47	16.27	15.82	9.59	14.16
0.47 "	17.10	17.44	15.76	12.84	15.26
0.20 "	18.95	15.99	15.24	10.50	14.88
Sig. diff. ($P = 0.05$)			1.47		0.89

until the second half of the season is reached. For potassium the interaction depends on the contrast between the figures for April 29 and the rest of the season; the first sample shows that the uptake was greatest at the lowest light intensity, although on subsequent sampling occasions the maximum was at the higher intensities (Fig. 3, Table IX).

It will also be noted in Figs. 3 and 4 that while at the end of the growth period the absolute amount of nitrogen in 1939 and phosphorus in 1938 declines in full daylight, uptake continues over this period in the reduced intensities. The same trend is shown, though less markedly, in the other data for nitrogen and phosphorus. Since the shaded leaves persisted for longer, the observed differences probably relate to an extended period of absorption at the lower light intensities.

From Figs. 3 and 4 it is equally clear that a reduction of light intensity has a far greater effect on dry weight increase than on uptake, and in consequence the percentage contents are considerably higher at the lowest light intensity than in full daylight (see Figs. 5 and 6). In 1938 there were significant interactions between light intensity and sampling occasion on the percentage contents of all elements, due to a larger effect of light in the final sample; in 1939 the interaction was significant only for potassium, and in this case it was due to a smaller effect in the last sample.

Table X shows quite clearly that when the only significant increase in dry weight is a small effect of additional nitrogen, the manurial effects of the three

TABLE X

Experiment III, 1938. The Significant Effects of Additional Mineral Nutrients on the Changes in Dry Weight and in the Composition of the Whole Plant

Means of all Light Intensities and Samples

Treatment.	% N.	% P_2O_5 .	% K.	Dry Wt. g.	mg. N.	mg. P_2O_5 .	mg. K.
Nitrogen	1.44	—	0.94	1.76	29.71	—	15.43
No nitrogen	1.32	—	0.89	1.69	25.99	—	14.10
Phosphorus	—	0.55	—	—	—	9.27	—
No phosphorus	—	0.50	—	—	—	8.45	—
Potassium	—	—	0.97	—	—	—	15.96
No potassium	—	—	0.86	—	—	—	13.57

elements on their total and percentage contents in the plant are very similar. Such similarity is to be expected, for the 'total amounts' have been obtained by multiplying the percentage content of each plant sample by its dry weight. In 1939, however (Table XI), some of the changes in the total amount of element in the plant are not reflected in the variations in percentage content,

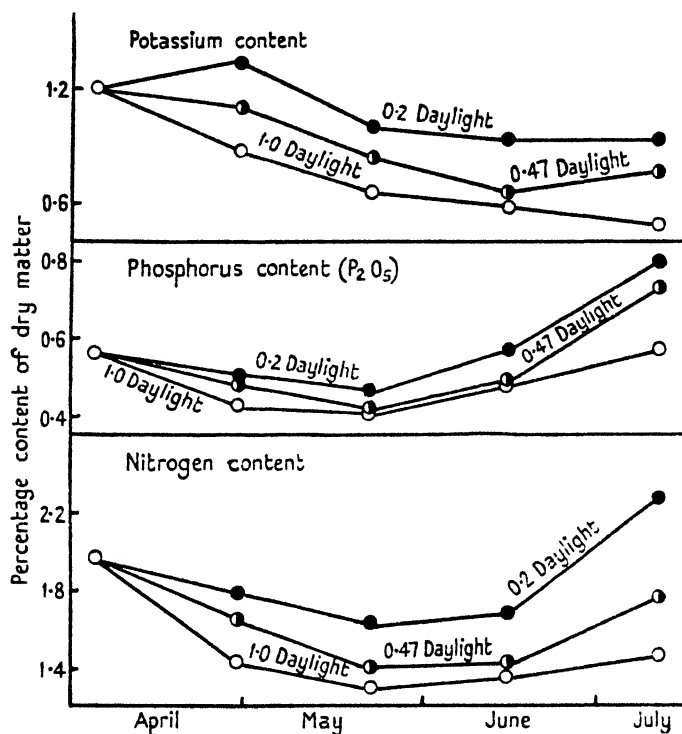


FIG. 5. The effects of varying light intensity on the seasonal changes in percentage content of nitrogen, phosphorus, and potassium within the whole plant (expt. III, 1938).

but have been brought about through the effects of nutrient supply in increasing plant weight. For instance, while additional nitrogen and phosphorus do not interact significantly on the percentage content of any of the three elements studied, the effect of each on the dry weight is significantly augmented by the presence of the other, and in consequence there are similar interactions on the total amounts of nutrients in the plant. In the case of total nitrogen content the interaction just falls short of significance, but the figures show that some degree of interaction has occurred. A similar interaction of phosphorus with potassium manuring acts on the total nutrient content through its effect on dry weight, rather than on the changes in percentage composition.

Only one significant interaction, that of nitrogen with potassium on the total potassium content of the plant, is not significantly reflected either in the dry weight changes or percentage potassium content: but both these quantities

show indications of an interaction, which is magnified sufficiently in their product to reach the level of significance $P = 0.05$.

In expt. III there were no effects of nutrients apart from nitrogen on either the percentage content or the total amount of nitrogen in the plant, except that

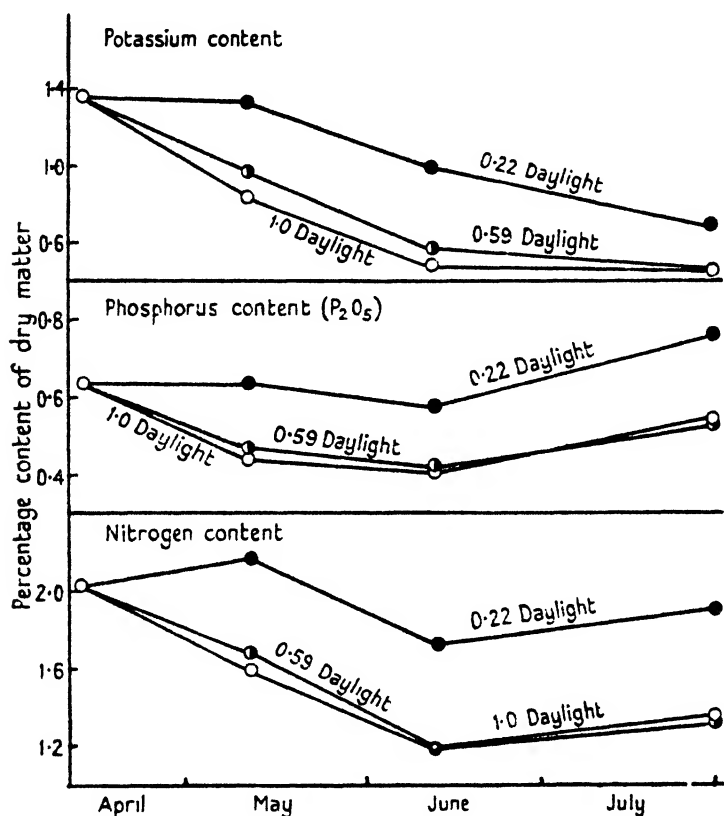


FIG. 6. The effects of varying light intensity on the seasonal changes in percentage content of nitrogen, phosphorus, and potassium within the whole plant (expt. IV, 1939).

potassium produced a higher final percentage content in the bulb (see Table XIII), although not at any other stage. This result was not again obtained in 1939.

In 1939 the percentage nitrogen content was increased by additional nitrogen only. But since nitrogen and phosphorus both caused increases in the dry weight, it is to be expected that they would also give rise to corresponding increases in the total nitrogen content of the plant (Table XI).

In 1938 the percentage phosphorus content was only affected by phosphorus supply, but in 1939 additional nitrogen also had a positive effect. As in the case of nitrogen, the various effects in 1939 on the plant weight were reflected in the results for total phosphorus content (Table XI).

In both expts. III and IV, besides the effect of potassium supply, additional

TABLE XI

Experiment IV, 1939. The Significant Effects and Interactions of Additional Mineral Nutrients on the Changes in Dry Weight and in the Composition of the Whole Plant

Means of all Light Intensities and Samples

Treatment.	% N.	% P_2O_5	% K.	Dry wt. g.	mg. N.	mg. P_2O_5 .	mg. K.
Nitrogen	1.81	0.56	0.80	5.34	90.80	28.82	38.69
No nitrogen	1.33	0.51	0.72	4.95	63.57	24.44	33.44
Phosphorus	—	0.58	—	5.36	80.94	29.82	37.49
No phosphorus	—	0.48	—	4.93	73.45	23.44	34.65
Potassium	—	—	0.96	—	—	—	45.85
No potassium	—	—	0.57	—	—	—	26.28
N and P	—	—	—	5.72	96.15*	32.99	41.89
No N and P	—	—	—	5.03	65.72	26.66	33.08
N, no P	—	—	—	4.97	85.47	24.65	35.49
No N, no P	—	—	—	4.92	61.42	22.21	33.80
N and K	—	—	1.08*	5.54*	—	—	49.71
No N, K	—	—	0.94	5.13	—	—	41.98
N, no K	—	—	0.58	5.14	—	—	27.67
No N, no K	—	—	0.55	4.81	—	—	24.89
P and K	—	—	—	5.72	86.24	31.32	48.65
No P, K	—	—	—	4.95	71.66	22.87	43.04
P, no K	—	—	—	5.02	75.63	28.32	26.32
No P, no K	—	—	—	4.94	75.23	23.99	26.24

* Interaction not significant.

nitrogen also had a significant positive effect on the percentage potassium content. Since there were negligible growth responses to increased nutrient level in 1938, the effects on the total content of potassium were the same as those on percentage content. There was also an interaction of additional phosphorus with occasion on the total amount of potassium in the plant (Table XII), but this is only just significant at the $P = 0.05$ level and depends on an apparent marked increase in potassium content on April 29 of the plants receiving additional phosphorus.

TABLE XII

Experiment III, 1938. The Interaction of Phosphorus Level and Sampling Occasion on the Mean Potassium Uptake

Potassium per Plant in Milligrams

	Apr. 4.	Apr. 29.	May 22.	June 15.	July 11.
Phosphorus	13.49	18.02	16.65	15.74	10.47
No phosphorus	13.50	16.32	16.49	15.46	11.49

Sig. diff. ($P = 0.05$) = 1.20.

In 1939 the combined effects of nitrogen and potassium on the percentage potassium content, together with the dry weight responses, produced not only positive effects for all three nutrients but significant interactions with one another on the total amount of potassium present in the plant (Table XI).

The interaction between light intensity and mineral nutrient supply on nutrient uptake

All the significant manurial effects on uptake, excluding interactions between nutrients, shown in Tables X and XI have been resolved into their component parts at each separate light intensity, and are presented in Figs. 7-12. In all cases, with one exception, the effects of increased nutrient supply on the percentage content of any of the elements are of much the same magni-

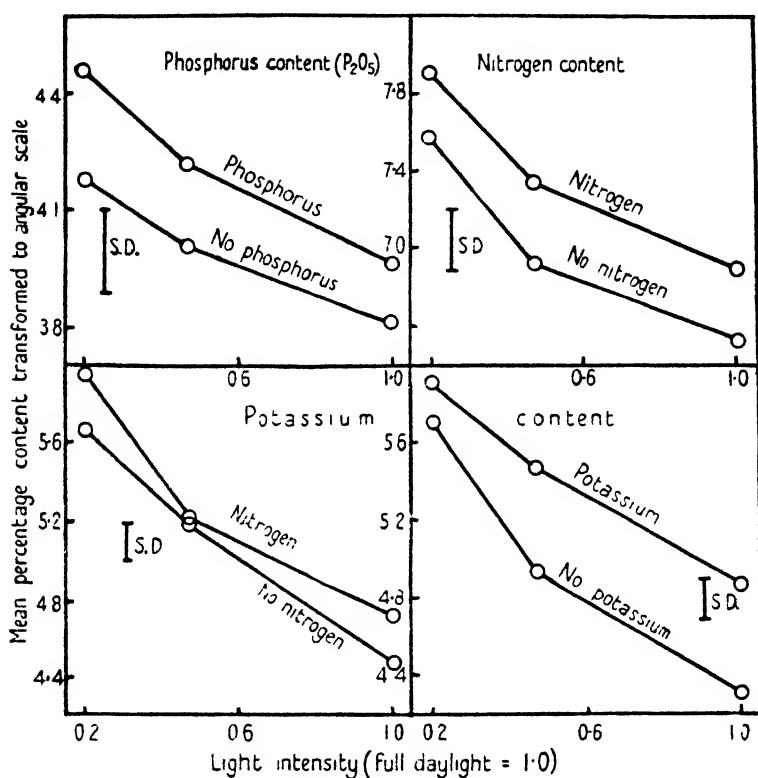


FIG. 7. The interrelationship between varying light intensity and changes in the level of mineral nutrient supply on the percentage content of nutrients within the whole plant (expt. III, 1938).

tude at all levels of light intensity, in so far that they are not less at the third than at the two higher light intensities. The exception is the effect in 1938 of additional potassium on percentage potassium content (Fig. 7); here the effect of potassium decreases as the light intensity is reduced; the interaction is significant, but there is no indication of such an effect in the following year (Fig. 9).

The interaction between light intensity and nutrient level on the uptake of each element by the plant differs according to whether there has been an effect of increased nutrient supply on percentage content, on the dry weight, or on both. Thus, when there is little or no manurial effect on dry weight, as

throughout 1938 and in the case of additional potassium in 1939, the effects on the total amounts of each element resemble those on the corresponding percentage contents: in neither case do they diminish with decreasing light intensity (Figs. 8 and 12). When there is an effect on dry weight, but not on percentage content, the gain in total content due to an increased nutrient supply resembles the change in dry weight in that it becomes negligible at the lowest light intensity, e.g. the positive effect of phosphorus supply on total

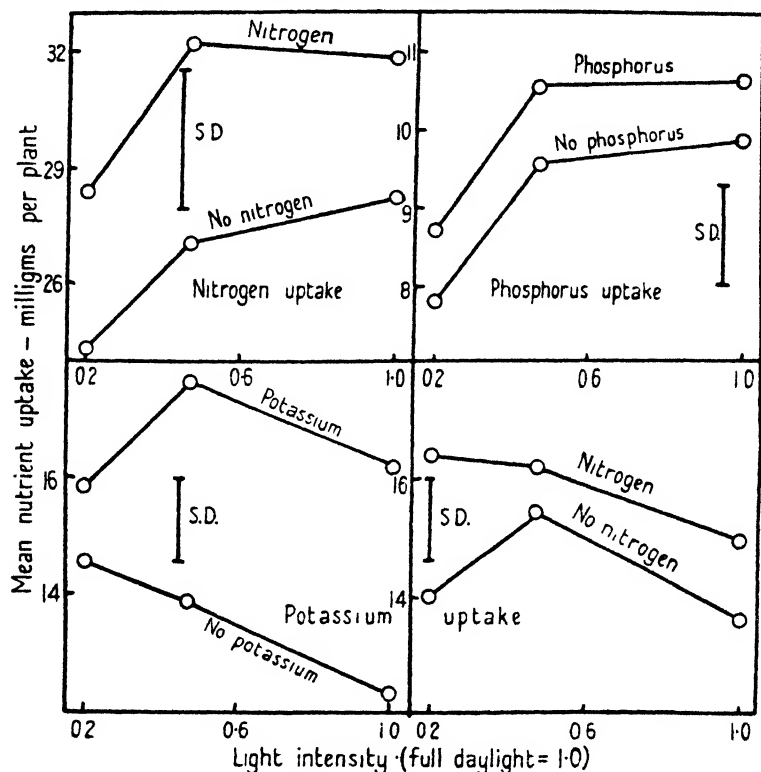


FIG. 8. The interrelationship between varying light intensity and changes in the level of mineral nutrient supply on the uptake of nutrients (expt. III, 1938).

nitrogen and potassium uptake in 1939 (Figs. 11 and 12). But when the nutrient supply level increases both the dry weight and the percentage content, there is an effect on uptake at all light levels, but this decreases as the intensity falls, e.g. the positive effects in 1939 of additional nitrogen on the uptake of all three elements, and of additional phosphorus on phosphorus uptake (Figs. 11 and 12).

The effects of varying light intensity and nutrient supply level on the distribution of mineral nutrients within the plant

Final composition of the bulb. The initial weight and percentage mineral nutrient content of the bulbs at the time of planting in the autumn, together with the comparable figures for the mature bulb in the following summer at

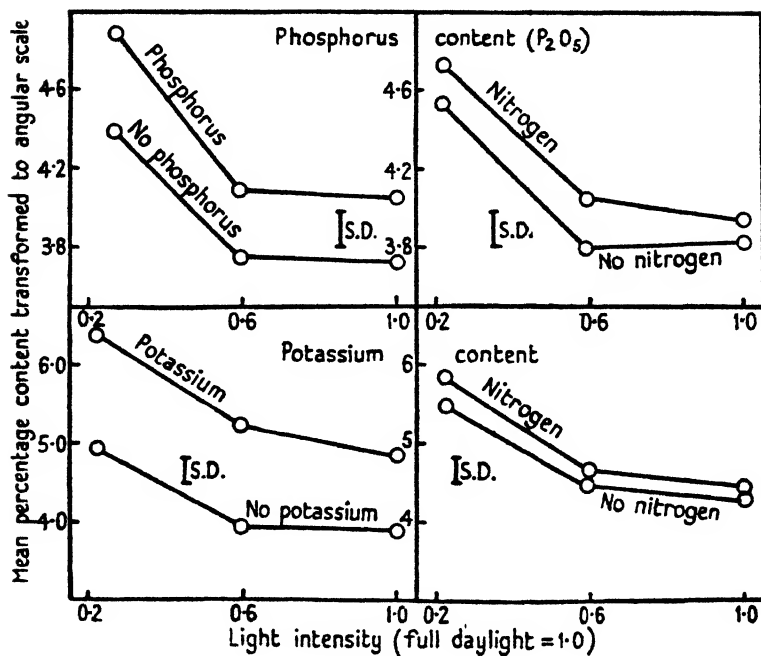


FIG. 9. The interrelationship between varying light intensity and changes in the level of mineral nutrient supply on the percentage content of phosphorus and potassium within the whole plant (expt. IV, 1939).

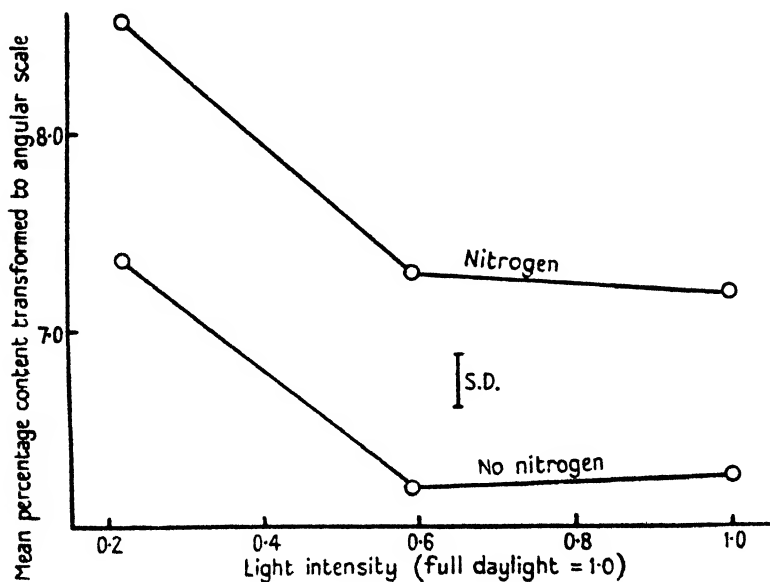


FIG. 10. The interrelationship between varying light intensity and changes in the level of nitrogen supply on the percentage content of nitrogen within the whole plant (expt. IV, 1939).

the end of the experiments, have been set out in Tables XIII and XIV. Since the means are obtained from one occasion rather than several, not all the effects which prove to be significant when the whole season is taken into account can be shown to be so for the mature bulb; this is particularly the case in 1938 (Table XIII). However, in one instance a significant effect appears which is not shown by the means for the whole season; namely, a positive effect of potassium supply on percentage nitrogen content.

TABLE XIII

The Effects of Mineral Nutrient Supply on the Composition and Weight of the Mature Bulb

Nutrient level.	Dry weight, g.	Mineral content, % of dry matter.		
		N.	P ₂ O ₅ .	K.
<i>Expt. III, 1938</i>				
Initial	1.06	1.92	0.53	1.18
Nitrogen	3.37	1.84	0.67	0.77*
No nitrogen	3.21	1.78	0.71	0.67
Phosphorus	3.22	1.84	0.72*	0.69
No phosphorus	3.36	1.78	0.66	0.75
Potassium	3.29	1.91*	0.72	0.78
No potassium	3.26	1.72	0.66	0.66
<i>Expt. IV, 1939</i>				
Initial	3.36	0.92	0.39	0.70
Nitrogen	5.90*	1.73*	0.65*	0.57*
No nitrogen	5.16	1.32	0.57	0.50
Phosphorus	5.70	1.56	0.64	0.52
No phosphorus	5.36	1.49	0.58	0.55
Potassium	5.86*	1.52	0.60	0.64
No potassium	5.20	1.53	0.62	0.44

* Pairs of figures differing significantly are marked with an asterisk.

TABLE XIV

The Effects of Light Intensity on the Composition and Weight of the Mature Bulb

Light intensity.	Dry weight, g.	Mineral content, % of dry matter.		
		N.	P ₂ O ₅ .	K.
<i>Expt. III, 1938</i>				
1.00 daylight	4.08	1.44	0.56	0.48
0.47 ,,	3.46	1.75	0.72	0.76
0.20 ,,	2.32	2.24	0.80	0.91
<i>Expt. IV, 1939</i>				
1.00 daylight	6.31	1.35	0.54	0.46
0.59 ,,	6.28	1.32	0.53	0.45
0.22 ,,	4.00	1.90	0.76	0.69

It will be observed in Table XV that the corresponding final percentage contents at the highest and lowest light intensities are approximately the same

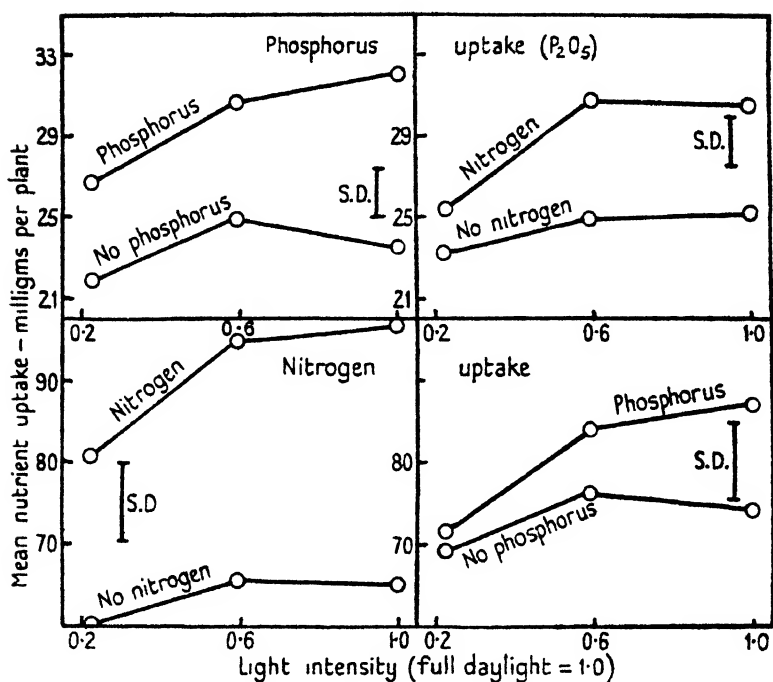


FIG. 11. The interrelationship between varying light intensity and changes in the level of mineral nutrient supply on the uptake of phosphorus and nitrogen (expt. IV, 1939).

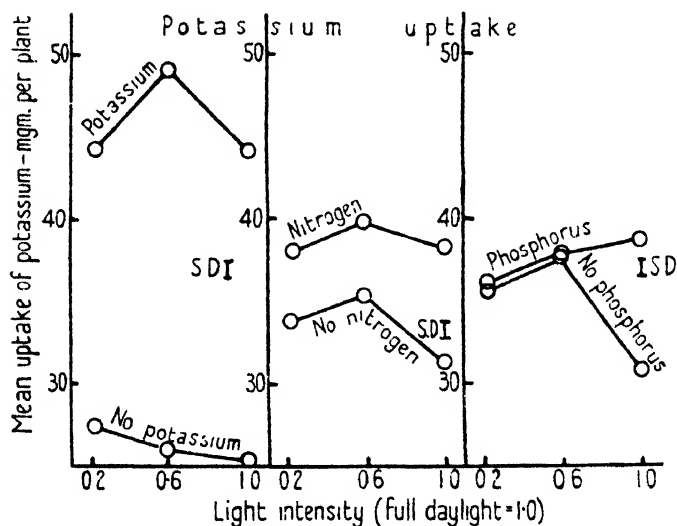


FIG. 12. The interrelationship between varying light intensity and changes in the level of mineral nutrient supply on the uptake of potassium (expt. IV, 1939).

in the two years, although the initial contents of the 1939 bulbs are much less than those of 1938. It is suggested that the low initial contents in 1939 may be the reason for the far greater responses to manuring in that year. Bulbs grown in the lower light intensities had, at the end of the season, a considerably higher percentage content of the three elements than bulbs grown in full daylight. In expt. III only at the lowest light intensity was the initial percentage content of nitrogen maintained to the end of the season, but in expt. IV there was an increase, irrespective of the intensity. In both years there was a seasonal gain in phosphorus content; whereas with potassium the percentage content in expt. III fell irrespective of the level of either light intensity or potassium supply. In expt. IV only a combination of 0.20 daylight and additional potassium led to the final content exceeding the initial value.

TABLE XV

The Interaction between Light Intensity and Mineral Nutrient Supply on the Final Composition of the Bulb

	Light intensity (daylight = 1.00)			Initial content.
	1.00	0.47-0.59	0.20-0.22	
<i>Expt. III, 1938</i>				
% nitrogen content				
Nitrogen	1.44	1.88*	2.21	1.92
No nitrogen	1.44	1.63	2.28	—
% P ₂ O ₅ content				
Phosphorus	0.58	0.74	0.85*	0.53
No phosphorus	0.54	0.70	0.74	—
% potassium content				
Potassium	0.54*	0.83*	0.97*	1.18
No potassium	0.43	0.69	0.85	—
<i>Expt. IV, 1939</i>				
% nitrogen content				
Nitrogen	1.58*	1.49*	2.13*	0.92
No nitrogen	1.13	1.15	1.63	—
% P ₂ O ₅ content				
Phosphorus	0.57*	0.53	0.82*	0.39
No phosphorus	0.51	0.52	0.71	—
% potassium content				
Potassium	0.55*	0.54*	0.82*	0.70
No potassium	0.37	0.37	0.57	—

* Pairs of figures differing significantly are marked with an asterisk.

Reference has already been made to the transference of mineral nutrients to the bulb at the end of the growing period. The effects of varying light intensity on this transference is seen in Tables XVI and XVII. As a general rule the lower the light intensity the larger is the relative rise in the bulb of

TABLE XVI

Experiment III, 1938. The Effects of Varying Light Intensity on the Withdrawal of Nutrient Elements from Leaf and Flower to Bulb

Light* intensity.	mg. N.		mg. P ₂ O ₅ .		mg. K.	
	June 15.	July 11.	June 15.	July 11.	June 15.	July 11.
1.00 daylight						
Bulb	28.63	28.51	10.04	11.08	11.55	9.59
Leaf	2.12	—	0.41	—	0.74	—
Flower	5.27	—	2.17	—	3.53	—
Whole plant	36.02	28.51	12.62	11.08	15.82	9.59
0.47 daylight						
Bulb	25.28	29.79	8.67	12.33	9.36	12.84
Leaf	3.41	—	0.67	—	1.50	—
Flower	6.36	—	2.39	—	4.90	—
Whole plant	35.05	29.79	11.73	12.33	15.76	12.84
0.20 daylight						
Bulb	17.44	26.04	6.19	9.26	8.94	10.50
Leaf	3.85	—	0.70	—	1.69	—
Flower	6.59	—	2.37	—	4.61	—
Whole plant	32.98	26.04	9.26	9.26	15.24	10.50

* The values of light intensity are the means for the whole season. Daylight = 1.00.

TABLE XVII

Experiment IV, 1939. The Effects of Varying Light Intensity on the Withdrawal of Nutrient Elements from Leaf and Flower to Bulb, and Transference from Flower to Seed

Light intensity.	mg. N.		mg. P ₂ O ₅ .		mg. K.	
	June 12.	July 31.	June 12.	July 31.	June 12.	July 31.
1.00 daylight						
Bulb	57.59	81.10	21.59	34.19	24.26	29.88
Leaf	12.17	—	2.59	—	2.04	—
Flower	14.42	—	4.84	—	8.68	—
Seed	—	8.15	—	2.76	—	0.87
Whole plant	84.18	89.25	29.02	36.95	34.98	30.75
0.59 daylight						
Bulb	58.68	84.55	22.66	33.60	25.76	29.28
Leaf	12.56	—	2.67	—	6.75	—
Flower	10.78	—	3.35	—	7.41	—
Seed	—	1.32	—	0.46	—	0.11
Whole plant	82.02	85.84	28.68	34.06	39.92	29.39
0.22 daylight						
Bulb	40.97	76.08	16.26	30.45	18.66	27.53
Leaf	20.51	—	4.15	—	15.33	—
Flower	8.37	—	2.90	—	5.45	—
Seed	—	8.12	—	1.02	—	0.25
Whole plant	69.85	79.20	23.31	31.47	39.44	27.78

nitrogen, phosphorus, and potassium between the penultimate and final samples. As to what proportion of these increases are due to transference from the leaves and inflorescence cannot be assessed with precision since at the time of the one from last sample, the plants under the different light intensities were at different phases of development. For example, it has already been recorded that plants in full daylight are the first to show signs of leaf senescence and that under the lowest light intensity nutrient uptake continues until later on in the season. Nevertheless, it is clear that even allowing for the nutrients lost in the seed there is never at any light level a complete withdrawal of the remainder to the bulb.

Composition of leaves and inflorescence. The percentage contents of leaf and flower fall gradually through the season but are invariably considerably higher than the corresponding figures for the bulb. This difference is particularly marked for the potassium contents, which may in the leaf reach a concentration four to six times that of the bulb. A reduction of light intensity increases the percentage mineral nutrient contents of the leaf (Tables XVIII and XIX), especially the potassium content, and since dry weight of the leaf is not affected by light intensity, the total amounts in the leaf of all three elements are also raised. The only exception is phosphorus in expt. III, when the apparent increase is not significant.

TABLE XVIII

Experiment III, 1938. The Effects of Varying Light Intensity on the Mineral Nutrient Content of the Leaf

Percentage of dry weight; means of samples May 22 and June 15

Light Intensity.	N.		P ₂ O ₅ .		K.	
	% content.	Angular scale.	% content.	Angular scale.	% content.	Angular scale.
1.00 daylight	3.05	10.03	0.64	4.28	1.35	6.60
0.47 "	3.28	10.41	0.62	4.86	1.70	7.38
0.20 "	3.46	10.69	0.67	5.11	1.76	7.57
Sig. diff.		0.32		not sig.		0.26
(<i>P</i> = 0.05)						

In expt. III, when the leaf data for the two occasions—May 22 and June 15—are analysed statistically, the increase in percentage nutrient content with reduction in light intensity are not significantly different for the two occasions. In expt. IV, however, when the relevant sampling dates were May 11 and June 12, there are significant interactions of light with occasion, the light effect being much more marked at the latter date.

Analyses of variance of percentage potassium content of the leaf in 1938 and percentage phosphorus content in 1939 also show that there are other significant effects. In 1938 both nitrogen and phosphorus increased the potassium content on May 22, but significantly decreased it on June 15; phosphorus supply also had a positive effect at a high light intensity, and a

negative effect at reduced intensities (Table XX). In 1939 nitrogen had a significant negative effect on the phosphorus content of the leaf; viz. 0.70 and 0.76 per cent. for plants with and without additional nitrogen.

TABLE XIX

Experiment IV, 1939. The Effects of Varying Light Intensity on the Mineral Nutrient Content of the Leaf

Light intensity.	N.		P ₂ O ₅ .		K.	
	May 11.	June 12.	May 11.	June 12.	May 11.	June 12.
Percentage content of dry weight						
1.00 daylight	4.27	2.57	0.85	0.56	2.60	1.31
0.59 "	4.07	2.65	0.81	0.58	2.72	1.32
0.22 "	4.08	3.36	0.90	0.68	3.02	2.55
Angular scale						
1.00 daylight	11.92	9.20	5.25	4.29	9.12	6.34
0.59 "	11.63	9.36	5.24	4.34	9.34	6.23
0.22 "	11.64	10.57	5.41	4.45	9.87	8.97
Sig. diff. ($P = 0.05$)	0.31		0.10		0.62	

TABLE XX

Experiment III, 1938. The Interactions of Additional Nitrogen and Phosphorus with Sampling Occasion, and of Additional Phosphorus with Light Intensity, on the Potassium Content of the Leaf

Percentages of dry weight					
N	May 22.	June 15.	P	May 22.	June 15.
	No N	No N		No P	No P
	2.01	1.19*		1.99	1.12*
	1.90	1.31		1.91	1.38
Light intensity (daylight = 1.0).					
				1.00.	0.47.
				0.20.	
P				1.42	1.55*
No P				1.70	1.81
				1.28	1.84

* Pairs of figures differing significantly are marked with an asterisk.

Two significant second-order interactions indicate that the interactions on percentage potassium content in 1938 of phosphorus with light intensity, and of potassium with light intensity, are dependent on sampling occasion. Similarly in 1939 there are significant interactions on percentage phosphorus content between nitrogen, phosphorus, and sampling occasion; phosphorus, potassium, and sampling occasion; and nitrogen, light intensity, and occasion. But since all include terms whose level of significance was very high, while the interactions themselves do not attain the $P = 0.01$ level, they do not, therefore, on statistical grounds, merit interpretation.

The effects of light intensity on the composition of the inflorescence are summarized in Table XXI. In many ways they reflect the same trends as

those in the leaves, although the changes are not so marked. Shading tends to increase the percentage content, and the most consistent increases are those for potassium.

TABLE XXI

The Effects of Varying Light Intensity on the Mineral Nutrient Content of the Inflorescence

Percentage content of dry weight.

Light intensity.	N.			P ₂ O ₅ .			K.		
	May 22.	June 15.	Mean.	May 22.	June 15.	Mean.	May 22.	June 15.	Mean.
<i>Experiment III</i>									
1.0 daylight	1.96	1.67	1.82	0.63	0.64	0.64	1.60	1.12	1.36
0.47 "	1.83	1.67	1.75	0.60	0.62	0.61	1.84	1.31	1.58
0.20 "	1.91	1.81	1.86	0.61	0.65	0.63	1.99	1.27	1.63
Angular scale									
1.0 daylight	8.04	7.42	7.73	4.57	4.56	4.56	7.25	6.03	6.64
0.47 "	7.76	7.41	7.58	4.43	4.51	4.47	7.79	6.56	7.17
0.20 "	7.93	7.71	7.81	4.49	4.62	4.55	8.09	6.46	7.28
Sig. diff.	0.37		0.18	n.s.		n.s.	0.28		0.31
(<i>P</i> = 0.05)									
	May 11.	June 12.	Mean.	May 11.	June 12.	Mean.	May 11.	June 12.	Mean.
<i>Experiment IV</i>									
1.0 daylight	2.65	1.89	2.27	0.90	0.64	0.77	1.67	1.12	1.40
0.59 "	2.40	1.78	2.09	0.85	0.55	0.70	1.49	1.22	1.36
0.22 "	3.10	1.89	2.00	1.13	0.64	0.89	1.93	1.22	1.58
Angular scale									
1.0 daylight	7.36	5.89	6.63	5.44	4.08	5.01	7.43	6.04	6.73
0.59 "	6.90	5.64	6.26	5.27	4.25	4.76	6.98	6.32	6.65
0.22 "	8.13	5.89	7.01	6.09	4.58	5.33	7.98	6.32	7.15
Sig. diff.	0.21		0.29	0.14		0.13	0.46		0.26
(<i>P</i> = 0.05)									

DISCUSSION

Although many investigations have been concerned with the effects of shading on the growth of plants, little critical consideration has been given to an examination of the relationships between light intensity, salt uptake, and the accumulation of the absorbed mineral nutrients within the plant. This hiatus is all the more surprising since the early workers had already indicated that heavily shaded plants contained a higher percentage of ash. For example, Pagnoul (1879, 1881) showed that covering sugar-beet plants with blackened cloches depressed growth, decreased the total amount of mineral nutrients in the plant, but increased the percentage content of ash. Similarly Thatcher (1909) found that potatoes, peas, and cereals when shaded with sacking during the flowering and ripening phases contained a greater percentage of ash than unshaded plants. It was also demonstrated by Pfeiffer, Blanck, and

Flügel (1912) that if the 'self shading' within a dense stand of oats was simulated by interposing a number of 'artificial plants' in a normal stand, then the percentage nitrogen content was increased.

Most of the subsequent papers, although they confirmed these earlier observations, did not add much detailed information. According to Kraybill (1923), shading with a layer of cotton cloth increased the percentage nitrogen content of shoots of both apples and peaches. Weissmann (1925) found that rye grown in pots in the north and south windows of a laboratory took up less phosphorus and potassium than potted plants grown out of doors. Domontowitsch and Groschenkow (1929) observed that decreasing the day length by 4-6 hours over 2-day periods depressed the uptake of nitrogen and phosphorus but had little effect on potassium uptake. Similar evidence was obtained by Panchaud (1934) that shading depressed the total amount of ash in radishes. With tomatoes grown under greenhouse conditions nitrogen absorption was not decreased until the light intensity fell below 0.5 of daylight (Porter, 1937).

More extensive data on the effects of shading on mineral uptake are to be found in the investigations of Mitchell (1934, 1936) and Gast (1937) working on the general problem of the light factor and the growth of coniferous seedlings. In the first of Mitchell's papers seedlings of *Pinus sylvestris* were harvested at the end of 100 days after having been grown at four levels of nitrogen supply and two levels of light intensity, namely, 0.87 and 0.50 of daylight. Under these conditions shading did not depress the uptake of nitrogen except at the highest level of nitrogen supply. A similar type of experiment (Mitchell 1936) was subsequently carried out with white pine (*Pinus strobus* ?), where the seedlings were subjected to four light intensities (daylight and 0.74, 0.53, and 0.29 of daylight) for 100 days. In this instance a reduction in the light intensity progressively decreased nitrogen uptake, had no effect on potassium uptake, and only depressed phosphorus absorption at the lowest light intensity. The effects of shading on the percentage mineral nutrient content of the seedlings appeared to be somewhat variable since the trends were indefinite and the experimental errors were not assessed. It would seem that the potassium content rose as the light intensity declined, but that the changes in nitrogen and phosphorus content were small.

Gast in his investigations was interested in the growth of *P. sylvestris* seedlings in 'raw' humus and sands under a light range of 0.04 to 0.5 of daylight. The effect of the lower light intensities in depressing nitrogen absorption was linked with the nitrogen availability in the pot culture medium since the greater the nitrogen supply the greater the depression of nitrogen uptake. Conversely the percentage nitrogen content of the plants rose as the light intensity fell. There was also a little evidence that phosphorus absorption at 0.22 of daylight was less than at 0.5 of daylight.

From these previous investigations it is evident that shaded plants tend to contain a higher percentage content of mineral nutrients and that the light factor operates differentially in the absorption of individual nutrients. The present experiments amplify and extend these earlier findings, since for the

first time the changes in the composition of the plant and the amount of nutrients absorbed have been periodically examined over the growing season. In addition the experiments have been designed on a multifactorial basis so that the effects and interactions between the light factor and nutrient level can be analysed by statistical methods.

Taking first the effects of light intensity on nutrient uptake by the plant, it is clear from Figs. 3 and 4 and Tables VII–IX that while a reduction of the

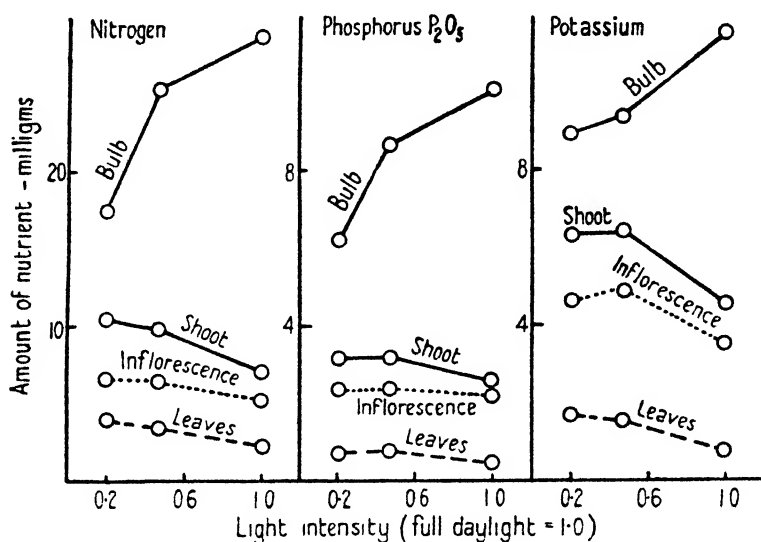


FIG. 13. The effects of varying light intensity on the partition of nitrogen, phosphorus, and potassium between the bulb plus roots and the shoot (leaves plus inflorescence) at the time of the fourth sampling occasion—June 15 (expt. III, 1938).

light intensity below half-daylight decreases the absorption of nitrogen and phosphorus it has no such effect on potassium absorption. In expt. III there is a progressive effect of shading in reducing the uptake of nitrogen and phosphorus as the season advances, but in expt. IV the seasonal effect is less evident.

It is also apparent from Figs. 8 and 11 that the decrease in nitrogen and phosphorus absorption brought about by shading is not dependent upon the external level of nutrient supply. On the other hand, in both experiments there is a significant interaction between potassium supply and light intensity since at the lowest light intensity additional potassium has had a smaller influence on potassium absorption (Figs. 8 and 12). In addition, in expt. IV an increase in the phosphorus supply has only increased potassium uptake in full daylight (Fig. 12).

It has already been pointed out (pp. 466–8) that the uptake of a nutrient element at various light levels is the product of the dry-matter production and the percentage content of the element within the tissues, and it is evident from Figs. 3 and 4 that the absorption of nitrogen, phosphorus, and potassium is

not proportional to the growth made at the various light levels. In fact a comparison of Figs. 3 and 4 with Figs. 7, 9, and 10 shows that while shading reduces growth it leads to a greater percentage content of nitrogen, phosphorus, and potassium within the tissues. It is therefore the balance between these opposing trends which conditions nutrient uptake at each light intensity.

In this connexion there is the further factor of the differential accumulation of nutrient elements within the plant. The effects of light intensity on the

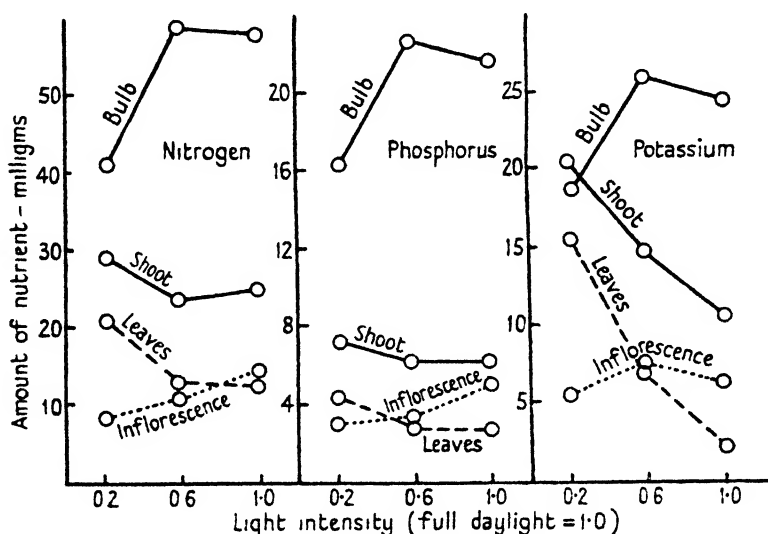


FIG. 14. The effects of varying light intensity on the partition of nitrogen, phosphorus and potassium between the bulb plus roots and the shoot (leaves plus inflorescence) at the time of the third sampling occasion—June 12 (expt. IV, 1939).

partition of nitrogen, phosphorus, and potassium between the bulb plus root and the shoot are seen in Figs. 13 and 14.

In selecting the most representative data there is the difficulty that the effects of varying light intensity on plant growth and nutrient uptake are not constant during the growth cycle. In expt. III (Fig. 13) the data refer to the fourth sampling occasion, since at this time the differences in plant weight and the uptake of nitrogen and phosphorus due to the light treatments are maximal (see Fig. 3). For similar reasons the third sampling occasion has been chosen in expt. IV (Fig. 14).

The choice of these periods is less satisfactory in the case of potassium absorption since the maximal uptake occurs earlier, more especially in expt. III, but nevertheless the differences are not large enough to invalidate these periods being selected so as to obtain a simultaneous comparison of the partition of nitrogen, phosphorus, and potassium between the shoot (aerial part) and the bulb (bulb and main roots). From both figures it is evident that shading leads to an accumulation of nutrients within the shoot and that this is most pronounced for potassium and least evident for phosphorus.

Moreover, the proportion in the shoot of the total potassium is higher than the corresponding relative amounts of nitrogen and phosphorus, and in consequence the effects of light intensity on potassium accumulation within the shoot are of greater consequence in determining total uptake by the plant. In fact, whereas the reduction in nitrogen and phosphorus absorption caused by a diminution in light intensity is largely a question of the amount accumulated by the bulb, in the case of potassium the absence of any positive effect

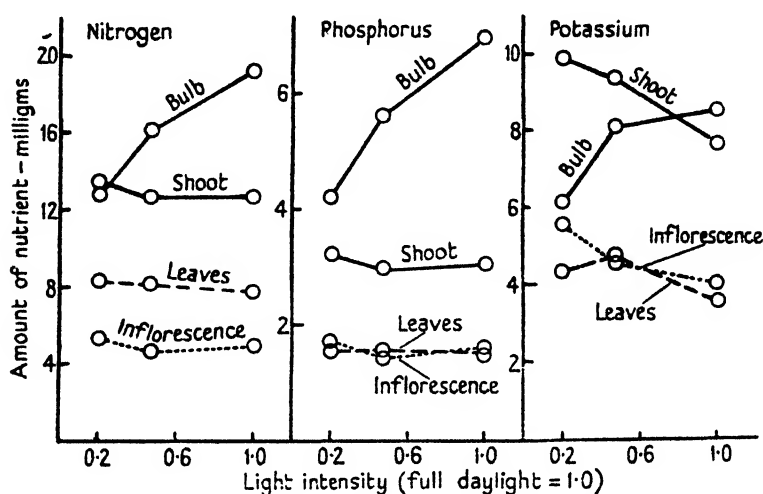


FIG. 15. The effects of varying light intensity on the partition of nitrogen, phosphorus, and potassium between the bulb plus roots and the shoot (leaves plus inflorescence) at the time of the third sampling occasion—May 22 (expt. III, 1938).

of shading in decreasing absorption is due to the accumulation within the shoot offsetting the decrease in the bulb.

The accumulation of each element within the bulb and shoot at the several light levels can again be subdivided into the effects on weight and on percentage content. In expt. III it has already been shown (Blackman and Rutter, 1947) that neither light intensity nor the addition of nutrients has any significant effects on either maximal leaf or flower production. It therefore follows that the data for shoot and leaves given in Fig. 13 largely reflect changes in percentage content. From Table XVIII it is also evident that the effects of shading on the leaf potassium content (mean of sampling occasions 3 and 4) are greater than those for nitrogen or phosphorus. These results are therefore in line with the observations of Tanada (1946), who found that in coffee plants subjected to 0.5 and 0.25 of daylight the rise in percentage potassium content of the leaves was greater than the increase in nitrogen, phosphorus, or calcium.

In considering the partition of mineral nutrients between the shoot and the bulb there is the further point that this is in part dependent on the sampling occasion. Whereas in expt. III the bulb and inflorescence weight increase during the growing season up till the fourth sampling, leaf-weight reaches

a maximum at the third sample. Moreover, with the earlier samples the percentage mineral contents are higher and in consequence the proportion of 'shoot' nutrients accumulated in the leaves is larger. This difference between occasions can be clearly seen by comparing Fig. 13 with Fig. 15.

The corresponding results are less easy to analyse in the case of expt. IV, since although light intensity had no significant effect on maximum leaf production, shading depressed the weight of the inflorescence while both nitrogen and potassium increased leaf weight. Nevertheless the nutrient effects exhibit no significant interaction with light intensity and it is again apparent from Fig. 14 that shading increases the proportion of nutrients accumulating in the shoot. It is evident that this accumulation is most marked for potassium. Table XIX also confirms that in the leaves the changes in potassium content caused by shading are more pronounced than those for nitrogen or phosphorus.

Although in the previous papers it has been shown that the effects of light intensity on total plant weight largely relate to changes in bulb size, the accumulation of each nutrient within the bulb is to some extent also linked with the percentage content. From Table XIV it is evident that in the mature bulb there are large increases in the percentage content of all three elements as the light intensity is reduced. These data are not, however, strictly relevant since it has already been stressed that the final contents will in part be made up of the mineral nutrients translocated from the shoot during senescence. More comparable data are the contents corresponding to the sampling occasions of Figs. 13-15 and these are given in Table XXII. It is seen that

TABLE XXII

The Effects of Varying Light Intensity on the Mineral Nutrient Content of the Bulb

Light intensity.	Percentage content of dry weight.					
	N.		P ₂ O ₅ .		K.	
	May 22.	June 15.	May 22.	June 15.	May 22.	June 15.
<i>Experiment III</i>						
1.0 daylight	0.96	1.25	0.34	0.44	0.43	0.51
0.47 "	1.00	1.28	0.35	0.45	0.50	0.48
0.20 "	1.13	1.48	0.37	0.53	0.53	0.76
	May 11.	June 12.	May 11.	June 12.	May 11.	June 12.
<i>Experiment IV</i>						
1.0 daylight	0.93	1.00	0.31	0.37	0.40	0.41
0.59 "	0.92	1.03	0.31	0.40	0.44	0.45
0.22 "	1.36	1.37	0.49	0.54	0.64	0.64

shading, as in the case of both the leaves and inflorescence (Tables XVIII, XIX, XXI), has increased the nutrient content of the bulb. The pattern is, however, somewhat different and the differences are less marked. The average increase is still largest for potassium followed by phosphorus and then nitrogen.

A comparison of Table XXII with Tables XVIII, XIX, and XXI also brings out the differences in the mineral content of the bulb, leaves, and inflorescence. If over all light intensities the percentage content of each element in the bulb is taken as unity, then for the two experiments the leaves contain on average respectively 3.1, 2.9, and 1.6 times as much potassium, nitrogen, and phosphorus as the bulb, while the corresponding figures for the inflorescence are 3.0, 1.6, and 1.5. With the changes in content produced by shading there is a tendency as the light intensity is reduced for these ratios to fall in the case of both the leaves and the inflorescence. These differences are greater in the inflorescence, but even so are not large. Taking expt. III as an example, in daylight the ratios of inflorescence to bulb contents for nitrogen, phosphorus, and potassium are 1.65, 1.61, and 2.96, while at the level of 0.2 daylight the corresponding ratios are 1.46, 1.44, and 2.73.

From the foregoing discussion it is evident that the differential effect of shading on the absorption of potassium against that of nitrogen and phosphorus is primarily related to the ability of the shoot to accumulate potassium as the light intensity falls. Total uptake is the product of plant weight and percentage content, and light intensity operates in reducing plant weight largely through the changes in bulb size. Therefore the ability of the shoot to accumulate potassium is an important factor in counteracting the reduction of potassium in the bulb where the diminution in size is not offset by a comparable increase in content due to shading.

In discussing the effects of light intensity on mineral nutrient uptake and partition within the plant it should be stressed that the present results have been obtained with a monocotyledenous bulbous perennial in which the leaf and flower primordia are laid down in the winter before active growth starts in the spring. Other as yet unpublished investigations indicate that the relative effects of shading on the growth of the root, stem, and leaves of annual plants are somewhat different from those of the bluebell, and further investigations are required to determine whether the present findings on the relationships between nutrient uptake and light intensity are of more general application.

Conversely the seasonal uptake of mineral nutrients by the bluebell is of interest since most of the previous investigations in this field have been concerned with annual and biennial plants of economic importance. Moreover, the majority of these investigators only estimated the nutrients in the aerial portion. The results of Figs. 1 and 2 and Tables III and IV demonstrate that there are major differences between the trends for potassium, nitrogen, and phosphorus. On the whole these divergences are similar to those recorded by McCalla and Woodford (1935) for the wheat shoot. Accumulation of nitrogen and phosphorus continues steadily during the growing period in contrast to the earlier maximum for potassium. The peak for wheat does not appear to be as early in the growth cycle as that of the bluebell.

Finally there remains the question of how far the present investigations can contribute to a proper understanding of the autecology of the bluebell. In

the first paper of this series (Blackman and Rutter, 1946) it was demonstrated that in deciduous woodland with a closed canopy the degree of shading was the major factor controlling the distribution of the bluebell. It was also established that in such woodland the mean light intensity between March and June was of the order of one-third daylight. It is therefore clear that the effects of light intensity and mineral nutrient supply obtained in these investigations are likely to operate in closed woodland. In comparison, therefore, with more open situations there will be a restriction in the ability of plants to absorb nitrogen and phosphorus while potential potassium uptake will not be affected. At first sight it might therefore be put forward that in soils with a low mineral status an increase in shade might not only have a direct effect on growth but would also adversely limit nutrient uptake. On the other hand, these investigations have demonstrated that heavily shaded plants contain a higher percentage content of all three elements. Such accumulations imply that the nutrients are in an excess and cannot be utilized for growth due to limitations of carbohydrate supply. On the other hand, an increase in percentage content caused by a reduction in light intensity does not necessarily imply that there is an internal surplus, since in shaded plants lignification is less, the cell walls are considerably thinner, and in consequence the proportion of 'non-metabolic' material is smaller. Nevertheless, in the case of nitrogen there is evidence that unelaborated nitrogen does accumulate in the leaves of shaded grasses (Blackman and Templeman, 1940). Moreover, in the second paper of this series it has been shown that at 0.2–0.22 daylight light is the overriding factor controlling growth, since whereas in full daylight additional nitrogen, phosphorus, and potassium all increased growth there were no such increases at the lower light level. The conclusion is therefore reached that in woodland the light factor operates directly on the growth of the bluebell rather than indirectly through nutrient uptake.

SUMMARY

The two previous papers in this series have been concerned with the combined effects of varying light intensity and different levels of mineral nutrient supply on the seasonal growth of the bluebell and statistical analyses have been made of the effects and interactions of these factors on the leaf area, the net assimilation rate, and the changes in bulb, leaf, and inflorescence weight during the growing period. This paper relates to the differences brought about by the same experimental treatments in the uptake of nitrogen, phosphorus, and potassium, and the partition of these elements within the plant during the annual cycle of growth.

Replicated multifactorial experiments of similar statistical design were carried out in two consecutive years; there were eight balanced manurial treatments, viz. C, N, P, K, NP, NK, PK, NPK, combined with three levels of light intensity, obtained by shading the plots with screens of either cotton cloth or perforated sheet metal. In the first experiment the three light intensities were daylight and 0.47 and 0.20 of daylight, while in the second

year the light levels were 1.0, 0.59, and 0.22 daylight. As soon as the shoots of graded dormant bulbs, planted in the previous autumn, appeared above ground in the following spring the screens were placed in position and a sample of plants taken from each plot. Each sample was divided into bulb plus roots, leaves, and inflorescence, dried at 100°C., and weighed. Subsequently three or four further samples were taken during the growing season and treated in the same way.

The nitrogen, phosphorus, and potassium contents were determined on each of the sub-samples of bulb, leaf, and inflorescence, amounting in all to some 3,200 analyses. The estimation of potassium involved modifications of a technique in which the silver in a potassium silver cobaltinitrite complex is estimated by titration with ammonium thiocyanate.

The uptake of the individual nutrients during the seasonal cycle of growth is markedly different. During the winter there is some absorption of all three elements followed by a rapid increase when the leaves have started to expand in April. In the case of potassium the uptake reaches a maximum by the time flowering commences and subsequently the plant slowly loses potassium. On the other hand, the absorption of phosphorus and nitrogen continues steadily until flowering has been completed and there is no loss until the onset of leaf senescence.

During the period when the seeds are ripening there is a considerable withdrawal of mineral nutrients from the shoot to the bulb. This transference is most effective for phosphorus and least effective for potassium. Under conditions favouring seed production more than half the nitrogen and phosphorus but only a tenth of the potassium found in the ripening inflorescences remain in the seeds and capsules.

The percentage contents of nitrogen, phosphorus, and potassium within the plant all reach a maximum prior to flowering. The leaves and inflorescences are far richer in nitrogen and potassium than the bulbs, but the differences in phosphorus content are much less marked.

Additional nitrogen, phosphorus, and potassium bring about corresponding rises in the percentage contents and the total amounts taken up by the plant. In the first experiment additional nitrogen increased the content and uptake of potassium. In the second experiment the only interactions for nutrient uptake which were *not* significant were nitrogen and potassium on both nitrogen and phosphorus uptake and nitrogen and phosphorus on nitrogen uptake. Not all the significant interactions for uptake were also reflected in the changes in percentage content since the additional nutrients increased uptake through the changes in plant weight rather than through changes in the percentage content.

The uptake of potassium, relative to that of nitrogen and phosphorus, is differentially affected by varying light intensity. Although at half-daylight there are no significant effects, a reduction of the light level to 0.2–0.22 of daylight causes a significant diminution in the absorption of nitrogen and phosphorus, but there is no such decrease in the case of potassium. These

reductions in uptake are only in part correlated with the effects of light level on the growth rate since the diminutions in uptake are less than the corresponding reductions in plant weight.

The influence of shading on nitrogen, phosphorus and potassium uptake is in part dependent on the level of nutrient supply. In one of the two experiments the increased absorption of phosphorus due to additional phosphorus was least at the lowest light intensity while there were also similar interactions between light intensity and phosphorus on potassium and nitrogen absorption while an increase in the nitrogen supply raised the uptake most in full daylight of all three elements.

Shading increases the percentage content of nitrogen, phosphorus, and potassium in the bulb and shoot. The increases are largest for potassium and least for phosphorus. The effects of light intensity on the percentage contents are relatively unaffected by the nutrient supply level. In one experiment the addition of phosphorus only increased the leaf phosphorus content in full daylight, while the gain in the potassium content of the whole plant due to increased potassium supply diminished with falling light intensity.

The differential effects of shading on uptake can be related to the varying ability of the shoot and the bulb to accumulate nutrients under the different light levels. Lowering the light intensity leads to an increase in the total amount of nitrogen, phosphorus, and potassium in the shoot but reduces the amounts found in the bulb. The accumulations in the shoot are most marked for potassium and are sufficient to offset the decreases in the bulb caused by shading, with the result that total uptake is not affected by light intensity. In the case of nitrogen and more particularly of phosphorus, the accumulations in the shoot are small compared to the losses in the bulb, and so nitrogen and phosphorus absorption is depressed by shading.

The partition of the nutrients within the plant is dependent upon the effects of light intensity on the growth of the bulb, inflorescence, and leaves coupled with the changes in content of the individual nutrients. Varying light intensity has no significant effect on maximum leaf production, causes only small changes in the development of the inflorescence, but produces large differences in bulb size. The accumulation of individual nutrients in the shoot under the reduced light intensity is therefore largely a question of changes in content, while the total quantities found in the bulb are dominated by decreases in size, though the increases in percentage content due to shading may be a contributory and opposing factor. Moreover, the differences in level of the actual percentage contents of leaves, inflorescence, and bulb must be taken into account. Thus, the high potassium content of the shoot, relative to the bulb, coupled with the marked effects of shading in increasing the percentage contents of potassium in both the bulb and shoot, all operate in minimizing the effects of shading on potassium uptake per plant.

Conversely the lower proportions of the total nitrogen and phosphorus in the shoot, together with the lesser influence of shading in increasing the percentage contents not only in the shoot but also in the bulb, do not offset the

reduction in total uptake operating through the decreases in bulb size brought about by shading.

Relating these investigations to the autecology of the bluebell it is clear from the earlier woodland studies that under the conditions of deciduous woodland with a closed canopy the degree of shading will be sufficient to bring about differences in mineral uptake and accumulation similar to those observed at the lowest light intensity of the present experiments. Such differences are, however, likely to play only a small part in controlling the distribution of the bluebell within these communities since the light factor rather than mineral nutrient supply is of major importance in determining the rate of growth.

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